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IN THE U.S. PATENT AND TRADEMARK OFFICE

Inventor	Zoltan GREFF et al
Patent App.	10/030,436
Filed	21 March 2002 Conf. No. 6522
For	2,3-BENZODIAZEPINE DERIVATIVES
Art Unit	1624 Examiner COLEMAN, B

Hon. Commissioner of Patents
Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL COMMUNICATION

This is in further response to the Office Action mailed 11 June 2004.

Applicants are submitting a signed Declaration Under 37 CFR 1.132 that corresponds to the unsigned declaration that was made of record on 12 October 2004 as an attachment to Applicants' amendment. The name of the declarant on the unsigned declaration was indicated as Zoltan Greff, the first listed inventor. However, the executed declaration was signed instead by Laszlo G. Harsing, MD, who is the person at assignee EGIS Gyogyszergyar RT most directly involved in carrying out the tests and in supervising the carrying out of the tests set forth in the declaration. Attached to the declaration is the full curriculum vitae of Dr. Harsing as

well as several publications to support the conclusions reached by Dr. Harsing in the declaration.

Favorable action is earnestly solicited.

Respectfully submitted,
The Firm of Karl F. Ross P.C.

By: Jonathan Myers, 26,963
Attorney for Applicant

December 1, 2004
5676 Riverdale Avenue Box 900
Bronx, NY 10471-0900
Cust. No.: 535
Tel: (718) 884-6600
Fax: (718) 601-1099

Enclosures: Executed 132 Declaration of Dr. Harsing

22096

IN THE U.S. PATENT AND TRADEMARK OFFICE

Inventor Zoltan GREFF et al
Patent App. 10/030,436
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For 2,3-BENZODIAZEPINE DERIVATIVES
Art Unit 1624 Examiner COLEMAN, B

Hon. Commissioner of Patents
Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.132

I, Laszlo G. Harsing, a citizen of Hungary, residing at 116 Bokenyfoldi ut, 1165 Budapest, Hungary, declare as follows:

THAT I have a number of years of experience in the preparation and testing of pharmaceutically active compounds in the treatment of neurodegenerative disorders;

THAT my full curriculum vitae may be attached hereto;

~~THAT I am an Applicant in U.S. Patent Application Serial No. 10/030,436 filed 21 March 2002 and directed to 2,3-BENZODIAZEPINE DERIVATIVES;~~

THAT in order to establish that the present application enables one "skilled in the art" to use the compounds of the

Formula (I) to treat a wide variety of neurodegenerative diseases in mammals, including humans, I have assembled the following background information and have either personally conducted or supervised the carrying out of the following tests:

BACKGROUND INFORMATION

The Examiner states that the specification does not provide enablement for the treatment of neurodegenerative diseases, therefore she rejects claims 16-27 under 35 USC 112, first paragraph on the grounds that the disclosure in the specification is non-enabling to permit one to use the new Formula (I) compounds to treat many of the neurodegenerative diseases. The Examiner states that neurodegenerative diseases cover a broad range of disorders with different etiology. She further states that established treatment plans for many neurodegenerative diseases are not treated by administering compounds similar as those of the Formula (I) disclosed in the present application. As an example, she mentioned Alzheimer disease, which is treated by administering acetylcholinesterase inhibitors.

1. Neurodegenerative diseases in general

Glutamate is the most abundant excitatory neurotransmitter in the brain. Glutamate receptors are categorized into ionotropic and metabotropic glutamate receptors. Ligand-gated ionotropic glutamate receptors are ion channels allowing cation flow into the neurons, which are categorized into the subgroups of NMDA, AMPA and kainic acid receptors [Dingledine, R. et al. (1999) The glutamate receptor ion channels. Pharmacol. Rev. 51, 7-61]. Excessive stimulation of ionotropic glutamate receptors, including AMPA receptors can cause neuronal degeneration and cell death called excitotoxicity whereby desensitization is delayed and calcium ion permeability is increased [Bennett, M.V. et al. (1996) The G1uR2 hypothesis: Calcium-ion permeable AMPA receptors in delayed neurodegeneration. Cold Spring Harb. Symp. Quart. Biol. 61, 373-384; A. Frandsen et at. (2003). AMPA receptor-mediated neurotoxicity: Role of calcium ions and desensitization. Neurochem. Res. 28(10) 1495-1499]. Delayed desensitization leads

to excess sodium and calcium ion entry into the cells.

Intracellular calcium ion concentration has a key role in the initiation of both apoptosis and necrosis.
AMPA receptor antagonists with 2,3-benzodiazepine moieties have been repeatedly shown to produce strong neuroprotective effect in several brain ischaemia models in laboratory species. Further, a great number of neurodegenerative diseases exists with different etiology that can be alleviated by administration of AMPA receptor antagonists with 2,3-benzodiazepine basic structure alone or in combination with other pharmaceutical agents. It is also important that despite the diverse and mostly unknown etiology of the neurodegenerative diseases (e.g. Alzheimer's disease, Parkinson's disease, Huntington's disease, Creutzfeld-Jacob disease, ALS, multiple sclerosis etc.), excitotoxicity is one of the common pathways causing substantial damage in the particular CNS areas involved in the given neurodegenerative disorder, thus antagonists or negative modulators or the excitatory neurotransmitter system could have beneficial effects in all such diseases.

2. Parkinson's disease

Parkinson's disease is a slowly progressing CNS disorder characterized by an accelerated loss of dopaminergic nerve cells in the substantia nigra due to an unknown neurodegenerative process. Symptoms appear first by the time the majority of dopaminergic neurons in the nigrostriatal system are already lost. The drawback of the currently applied therapy comprising administration of levodopa (and/or carbidopa) is that on the long term, most of the patients develop dyskinesia. Corticostriatal and thalamostriatal glutaminergic excitatory pathways stimulating both NMDA and AMPA receptors are also involved in the neuronal organization of movement and seem to contribute to levodopa-induced dyskinesia. Furthermore, ligand binding was increased in the putamen of parkinsonian patients experiencing motor complications compared to those who did not, suggesting that glutamate receptor supersensitivity in the putamen plays a role in the development of motor complications

[Dingledine, F. et al. (1999) The glutamate receptor ion channels. Pharmacol. Rev. 51, 7-61].

The role of AMPA receptors in the development of motor complications in monkeys rendered parkinsonian was studied in details [Konitsiotis, S. et al. (2000) AMPA receptor blockade improves levodopa-induced dyskinesia in MPTP monkeys. Neurology 54, 1589-1595]. Study findings showed that LY300164, a selective, non-competitive AMPA receptor antagonist potentiated the effects of low-dose levodopa on motor activity and decreased levodopa-induced dyskinesia. These results suggested that non-competitive AMPA receptor antagonists could be useful in the treatment of Parkinson's disease by enhancing the antiparkinsonian effects of levodopa and decreasing dyskinesia [Konitsiotis, S. et al. (2000) AMPA receptor blockade improves levodopa-induces dyskinesia in MPTP monkeys. Neurology 54,1589-1595].

Results of a pilot study of 30 patients with Parkinson's disease conducted at six US institutes and Europe indicate that talampanel, the active enantiomer of a compound with 2,3-

benzodiazepine structure, may have a major role in decreasing levodopa caused dyskinesia. Currently, the Parkinson Center of the Department of Neurology at the University of Miami (US) conducts a study on talampanel as a treatment for dyskinesia [www.parkinson.org].

Furthermore, improvement of akinesia was observed in rats with bilateral substantia nigra pars compacts lesion after administration of AMPA antagonists [Stauch, S.B. et al., (1995) Centrally administered AMPA antagonists increase locomotion in parkinsonian rats. J. Neural Transm. Park. Dis. Dement. Sect. 9, 145-149].

3. Multiple sclerosis

Multiple sclerosis is an autoimmune disease of the central nervous system that results in progressive fall of sensory and motor functions due to destruction of the myelin sheet of axons, thus resulting in neuronal death. It has been shown that glutamate receptors including AMPA receptors are present in oligodendrocytes and these cells are highly sensitive

to glutamate-induced excitotoxicity [Mature, C. et al. (2001) There is a link between excitotoxic oligodendroglial death and demyelinating diseases. Trends Neurosci. 24, 224-230]. The experimental method EAE (experimental autoimmune encephalomyelitis) induced in laboratory animals can be used as animal model for multiple sclerosis. It has been shown that daily treatment with an AMPA antagonist result in remarkable improvement of motor disability induced by EAE in rats and mice in a dose-dependent manner, rescuing as much as 60% of oligodendrocytes destroyed by EAE. Further, loss of motor neurons in the ventral horn of the lumbar spinal chord was attenuated [Smith, T. et al. (2000) Autoimmune encephalomyelitis ameliorated by AMPA antagonists. Nat. Med. 6, 62-66]. These results suggest that glutamate induced excitotoxicity mediated by AMPA receptors contributed to oligodendrocyte loss and motor disability and indicate the usefulness of AMPA antagonists in order to reduce neurological disability [Moga D. et al. (2002) Parvalbumin-containing interneurons in rat hippocampus have an

AMPA receptor profile suggestive of vulnerability to excitotoxicity. J. Chem. Neuroanat. 23, 249-253].

EXPERIMENTAL SECTION

4. Global cerebral ischaemia

Neuroprotective effects of AMPA antagonists according to the present invention were studied in transient global ischaemia model in gerbils. Transient global ischaemia of the brain induces delayed damage to neurons including hippocampal pyramidal cells in the CA1 sub-field and hilar neurons in the dentate gyrus and the striatal caudate-putamen region. The mongolian gerbil is a suitable animal for this model since due to defective brain circulation, bilateral carotid occlusion produces complete forebrain ischaemia in this species.

4.1. Methods

Male mongolian gerbils (60-70 g) were subjected to global ischaemia via bilateral common carotid artery occlusion (BCO) for 3 minutes under ether anaesthesia. During surgery, the

body temperature of the animals was kept at the individual preoperative levels. The compounds of the invention were administered at 20 mg/kg dose intraperitoneally, 45 minutes after reperfusion.

Four days after surgery the animals were deeply anaesthetised with pentobarbital (60 mg/kg i.p.) and perfused through the heart by a fixative solution containing 0.1 % glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. Brains were removed, post-fixed in the same solution overnight and 60 μm thick coronal sections were prepared. Alternate coronal sections of brains were stained by silver impregnation according to. Gallyas et al [details [Gallyas, F. et al. (1980) A reliable and sensitive method to localize terminal degeneration and lysosomes in the central nervous system. Stain Technol. 55, 299-306]. Neuronal damage in the hippocampal CA1 region was scored from 0 to 6 as follows: (0) undamaged; (1) <10%; (2) 10-30%; (3) 30-50%; (4) 50-70%; (5) 70-90%; (6) >90% cell loss.

In case of asymmetrical damage in the two sides, the rating was assigned to the higher score. Neurons were considered to be irreversibly damaged when they were shrunken and strongly argylophilic, somata of intact neurons appeared dark yellow. Differences among groups were statistically evaluated by Kruskal-Wallis ANOVA followed by Mann-Whitney U-test.

4.2. Results

Table 1 summarizes the neuroprotective effect of the compounds according to the present invention. According to results summarized in Table 1, compounds according to the present invention protected against neuronal death in the CA1 area of the hippocampus of gerbils subjected to 3 min global cerebral ischaemia. These results suggest that compounds of the present invention can be suitable for the treatment of neurological consequences of global cerebral ischaemia, occurring in hypotension (blood loss), circulatory collapse during sever injury or due to cardiac arrest followed by resuscitation, cardiac surgery with extra-corporeal circulation etc.

Table 1

Neuroprotective effects of the compounds of the Formula (I) according the present invention (20 mg/kg i.p.) in the CA1 area of mongolian gerbils subjected to 3 min bilateral carotid occlusion

Compound	Reduction in CA1 pyramidal cell death at day 4 after BCO
Example 27	51 %**
Example 28	45 %*
Example 30	47 %**

*p<0.05; ** p<0.01

5. Amyotrophic lateral sclerosis

The neuroprotective effects of the compounds of the invention in amyotrophic lateral sclerosis (ALS, motoneuron disease) was studied in cultured rat motor neurons against domoic acid induced neuronal death in vitro.

ALS is a severe neurodegenerative condition leading to relatively selective destruction of cortical, bulbar and spinal

motor neurons. Glutamate induced excitotoxicity is one of the mechanisms contributing to the development of motoneuron disease.

5.1. Methods

Motoneurons were purified using a combination of density gradient centrifugation and immune-purification according to Henderson [Henderson at al. (1995). Survival of newly postmitotic motoneurons is transiently independent of exogenous trophic support. J. Neurosci. 15(4), 3128-37]. Spinal cords were dissected from day E14.5 Sprague- Dawley rat embryos (Elevage Janvier, France). The largest cells were isolated by centrifugation on a 6.5 % (w/v) metrizamide density gradient. The immonoaffinity purification step used previously was replaced by a cell sorting step using microbeads (Arce V. et al. (1999) Cardiotrophin-1 requires LIFRalpha to promote survival of mouse motoneurons purified by a novel technique [J. Neurosci. Res. 55(1) 119-26]. Cells were incubated with a mouse antibody (anti-rat p75 antibody [Ig192)]. Subsequently motoneurons were incubated with magnetic microbeads conjugated to anti-mouse

secondary antibodies, thus allowing the purification of motoneurons on separating columns (Miltenyi Biotech Inc.). Cells were centrifuged through a BSA cushion and resuspended in complete medium (neurobasal medium supplemented with B27 (Life Technologies), 2% horse serum, 25 μM 2-mercaptoethanol. Cells were plated onto 384-well dishes coated with polyornithine and laminin in complete medium. Final volume was 100 µl in each well in the presence of 1 ng/ml brain-derived neurotropic factor (BDNF, R&D Systems).

After four days of culture (time for the upregulation of AMPA-kainate type glutamate receptors as the neurons mature) half of the medium was removed and replaced by a freshly made medium containing compounds. One hour later the cells were treated with domoic acid (10 µM final). Domoic acid, an AMPA-kainate receptor agonist was used instead of glutamate as it elicits non-desensitizing responses at AMPA receptors. The concentration of the domoic acid was optimized in preliminary experiments. Controls included (all with BDNF) no treatment, domoic acid treatment (10 µM final), domoic acid together with the compound

of Example 27 according to the present invention at different concentrations. Eight replicates were performed for each setting.

50 µl of the freshly prepared solutions were added to each well. Equal volume of the appropriate diluent was added to the controls.

The number of surviving motoneurons was counted 2 day later. Live cells were counted by an automated image analyzer (Trophos) after labelling with a vital dye, calcine-AM (Fluke). Results were analyzed using Student t-test (two-tailed, unpaired).

5.2. Results

The results of the study are summarized in Table 2. According to these results, the compound of Example 27 according to the present invention protected against domoic-acid induced cell death in cultured rat motoneurones in a concentration-dependent manner. It produced marked effect at the concentration

of 10 μM suggesting that compounds according to the present invention can be suitable for the treatment of ALS.

Table 2. Survival of cultured rat motor neurons (survival, expressed as % of the control group) after domoic acid (DA) induced cell death and treatment with different concentrations of the compound of Example 27 of the present invention.

Treatment	DA+0 μM	DA+0.1 μM	DA+1 μM	DA+10 μM	DA+100 μM
Survival	24 %	22 %	53 %	80 %***	96***

6. Stroke

Permanent or transient middle cerebral artery occlusion (MCAO) performed both in rats and mice is used as an animal model to mimic conditions that occur the in human brain during stroke.

6.1. MCAO in mice

6.1.1. Methods

Focal cortical ischaemia was produced by electrocoagulation of the left middle cerebral artery (MCA). Male MRI mice (30-35 g, Charles-River Hungary Ltd.) were anaesthetised with 2,2,2-tribromoethanol (500 mg/kg i.p., 10 mg/kg). An incision was made in the left temporoparietal region of the head between the orbit and the ear. The temporal muscle was incised and reflected forward. A small burr hole was drilled into the lateral outer surface of the skull just over the MCA and the stem of MCA was occluded by electrocoagulation. Compounds were administered intraperitoneally 15 minutes before MCA occlusion. Two days later animals were anaesthetised deeply with pentobarbital (100 mg/kg i.p., 10 ml/kg), perfused through the heart with 4% solution of 2,3,5-triphenyltetrazolium chloride. Animals were decapitated, brains were removed and placed in saline containing 8% formaldehyde solution for 24 hours. The necrotic (non-stained) area was determined by means of image analysis (DigiCell for Windows 4.0). Results were expressed as means±SEM for the treatment groups and statistical significance was assessed using ANOVA followed by Duncan test.

6.1.2. Results

Results are summarized in Table 3. According to these results, compounds of the present invention decrease cerebral infarct size after focal cerebral ischaemia in mice and produced strong neuroprotective effect with low minimum effective doses. Results suggest that the compounds of the Formula (I) of the present invention can be suitable for the treatment of human stroke.

Table 3.

Reduction of cerebral infarct size after focal cerebral ischaemia in mice

Compound	Minimum effective dose, mg/kg
Example 28	0.3
Example 34	3
Example 38	3

6.2. MCAO in rats

6.2.1. Methods

Permanent focal ischaemia was produced by electrocoagulation of the left MCA according to Brint et al. [Brint S. et al, (1988) Focal brain ischaemia in the rat: methods for reproducible neocortical infarction using tandem occlusion of the distal middle cerebral and ipsilateral common carotid arteries. J. Cereb. Blood Flow Metab. 8, 474-485]. Male Sprague Dawley rats (180-220 g) were anaesthetised with pentobarbital (60 mg/kg i.p.). The temporal muscle was incised and a 2 mm burr hole was drilled 2-3 mm rostal to the fusion of the zygomatic arch with the squamosal bone, exposing left MCA followed by MCA occlusion by electrocoagulation. The left common carotid artery was isolated and simultaneously occluded by bipolar electrocoagulation. Compounds were administered 30 minutes after MCA occlusion. After 48 hours, animals were deeply anaesthetised with pentobarbital (100 mg/kg perfused through the heart with 4% 2,3,5-triphenyltetrazolium chloride. Animals were decapitated, brains were removed and placed in saline containing 8% formaldehyde solution for at least 24 hours. Each brain was

sliced into 1 mm sections including the necrotic tissue. Area of necrosis was measured in each slice using a morphometric software (DigiCell). From the area of infarcts, an estimate of the hemispheric extent of ischaemic damage expressed in volume units was calculated. Results were expressed as mean±SEM for the treatment groups and statistical significance was assessed using ANOVA followed by Duncan test.

6.2.2. Results

It is concluded that compound of Example 28 decreased cerebral infarct size after focal cerebral ischaemia in rats and produced strong neuroprotective effect in an effective dose as low as 0.03 mg/kg and in a dose dependent manner. Results suggest that compounds according to the present invention could be suitable for the treatment of human stroke.

7. Cystic Periventricular Leukomalacia

Injection of S-bromo-willardiine or ibotenate on post-natal day 5 (P5) in mice induces neuronal death leading to

cortical brain lesions that mimics several aspects of human cystic periventricular leukomalacia, which is observed most frequently in premature human infants [M, Largeron et al. (2001). The neuroprotective activity of 8-alkylamino-1,4-benzoxazine antioxidants Eur. J. Pharmacol. 424(3) 189-94].

7.1. Methods

At postnatal day 5, Swiss mouse pups were anaesthetised for intracerebral (i.c.) and intraperitoneal (i.p.) injections. I.c. injections were performed using a 26-gauge needle mounted on calibrated microdispenser. The needle was inserted 2 mm under the external surface of scalp skin in the frontoparietal area of the right hemisphere 2 mm from the midline in the lateral-medial plane and 3 mm in the rostro-caudal plane from the juction between sagittal and lambdoid sutures. Two 1 µl boluses were injected at a 30 second interval. Fifteen micrograms of S-bromo-willardiine diluted in phosphate-buffered saline (PBS) was injected i.c.

Immediately following the i.c. injection of the excitotoxin, compound of example 27 of the present invention dissolved in PBS containing 10% dimethyl-sulfoxide (DMSO) was administered intraperitoneally at a dose of 1, 3 or 10 mg/kg. Controls received the solvent (9:1 PBS-DMSO mixture) alone.

Five days later pups were sacrificed and brains fixed in formaldehyde solution. Coronal serial sections in 15 μm thickness were cut and each third section was stained with cresyl-violet. Brains were completely and serially sectioned from the frontal pole to the occipital lobes permitting an accurate and reproducible determination of the maximum sagittal frontooccipital diameter of both the cortical plate and white matter lesions. This parameter was used as a reliable and reproducible index of the lesion size. Statistical analyses were performed with Student t-test and one-way ANOVA. When group interaction was found to be significant, Dunnett multiple comparison test was performed. Results were expressed as mean±SEM.

7.2. Results

It is shown that compound according to Example 27 of the present invention protected against S-bromo-willardiine induced lesions both in the cortical layers and the white matter in newborn mice at a minimum effective dose dose of 1 mg/kg and 1 mg/kg, respectively and produced strong neuroprotective effect. The results suggest that the compounds of the Formula (I) according to the present invention can be successfully used for the treatment of human cystic periventricular leukomalacia.

THAT based upon the background information and experimental data presented above, I conclude the following: The compounds of the Formula (I) of the present invention are non-competitive AMPA receptor antagonists. Experimental results obtained from animal model experiments indicate that compounds of the present invention possess strong neuroprotective effect, which is useful for the treatment of conditions and diseases of the central nervous system with seemingly very different etiology. In particular, the compounds of the Formula (I) of the present invention demonstrated good neuroprotective activity in generally accepted animal models of stroke, Parkinson's disease,

multiple sclerosis, amyotrophic lateral sclerosis and cystic periventricular leukomalacia, based on AMPA receptor antagonism. The mechanism whereby AMPA receptor antagonists prevent neuronal cell death in very different disorders is the inhibition of glutamate-induced excitotoxicity, which is a major mechanism leading to apoptosia and necrosis of nerve cells.

The experimental data presented hereinabove provide support for the establishment of utility of the compounds according to the present invention in several neurodegenerative disorders.

In summary, the compounds of the Formula (I) according to the present invention have surprisingly advantageous pharmacokinetic and metabolic properties, which result in a preferable pharmacological and toxicological profile as well as in increased patient compliance.

THAT I am aware of no information inconsistent with that presented above or which would lead one to a contrary conclusion; and

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further
THAT these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 11/24/2004 Signed: Laszlo G. Harsing

Laszlo G. Harsing, MD, PhD, DSc

CURRICULUM VITAE

Name: Dr. Laszlo Gabor Harsing

Date and Place of Birth: September 9, 1947, Budapest, Hungary

Citizenship: Hungarian

Marital Status
Married, Elizabeth Veinperl-Harsing
Pharmacist

Present Employment
Vice Director, Head of Division
Division of Preclinical Research
EGIS Pharmaceuticals Ltd

Education
1966: Graduated from secondary school

1972: Medical degree obtained with qualification of "Summa cum Laude" at the Semmelweis Medical School, Budapest

1984: Ph.D. degree obtained in neuropharmacology
Thesis: Regulation of Cholinergic Neurotransmission in the Striatum
Hunagrian Academy of Sciences, Budapest

1992: Doctor of Sciences (D.Sc.) degree obtained in neuropharmacology
Thesis: The Role of Heterogenous Alpha-2 Adrenoceptors in the Regulation of Noradrenergic Neurotransmission
Hunagrian Academy of Sciences, Budapest

1994: Lecturer in Pharmacology, degree obtained at the Department of Pharmacology, Semmelweis Medical School, Budapest

Employment in Hungary
1968-1972: Teacher in Physiology, Department of Physiology, Semmelweis Medical School, Budapest

1972-1981: Assistant Professor of Pharmacology, Department of Pharmacology, Semmelweis Medical School, Budapest

1981-1986: Research Fellow and Senior Research Fellow, Department of Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest

1986-1995: Associate Professor of Pharmacology, Department of Pharmacology Postgraduate Medical School, Budapest

1992-2000: Head, Department of Neurobiochemistry
Vice Director of Biological Research
Institute for Drug Research, Ltd., Budapest

2000-at present: Vice Director and Head
Division of Preclinical Research
EGIS Pharmaceuticals Ltd

Employment in the United States of America

1980-1981: Visiting Scientist, Fogarty International Fellowship, Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Washington, D. C., USA, Head: Dr. Erminio Costa, 20 months

1984-1985: Visiting Fellow, Department of Anesthesiology, Albert Einstein College of Medicine, New York, NY, USA, Head: Dr. Derick D. Duncalf, 13 months

1989-1992: Research Scientist, Center for Neurochemistry, The Nathan S. Kline Institute for Psychiatric Research, Rockland Psychiatric Center, Orangeburg, NY, USA, Head: Dr. Abel Lajtha, 36 months

1995-1996: Visiting Research Scientist, Fogarty International Fellowship, Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA, Head: Dr. Michael J. Zigmond, 20 months

Short-Term Fellowships

1975: Department of Pharmacology, 1st Medical University, Moscow, USSR

1976: Institute of Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

1977: Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

1982: National Institute of Mental Health, Washington, D.C., USA

1987 Department of Pharmacology, University of Geneva, Geneva, Switzerland

1988 Universidad Central de Venezuela, Caracas, Venezuela

1993 Nathan Kline Institute for Psychiatric Research, Orangeburg, NY, USA

1998 Department of Neurology, University of Pittsburgh, Pittsburgh, PA, USA

Research Activity
Publications in journals: 93

Chapters published in textbooks: 37

Abstracts appeared in journals: 26

Abstracts presented in scientific meetings: 95

Number of citation: 1170, January 2003

Cumulative impact factor of publications: 206.470, January, 2003

Teaching Experience
1970-1972: Teaching of physiology for medical students

1972-at present: Teaching of pharmacology for medical students and in postgraduate courses

Research Interest
Pharmacology, Neuropharmacology, Neurochemistry, Neurotransmitter release and interactions, Pharmacology of Transporters

Experimental Procedures Employed
Brain slice techniques, Isolated organs, Microdialysis and HPLC analysis, Measurement of radiolabeled and endogenous neurotransmitter release

Membership
Society for Neuroscience, USA
British Pharmacological Society
European Society for Neurochemistry
Hungarian Pharmacological Society
Hungarian Physiological Society

Journal Editorial Advisory Board, Member at
Neurochemical Research
Neurochemistry International

Honour
Bela Issekutz Lecture and Medal
Budapest, 2001

Reference
Dr. Henry Sershen, Center for Neurochemistry, The Nathan Kline Institute for Psychiatric Research, Orangeburgh, NY, USA. Tel.: 1-914-398-5530

Dr. Laszlo G. Harsing, Jr.

LIST OF PUBLICATIONS

1. RESEARCH ARTICLES

1. Hársing, L., Hársing, L., Jr., Bartha, J.: Uj szempontok az intrarenális haemodynamikában. Orvostudomány, 1972, 23, 295-301.

2. Kover, G., Harsing, L. G., Harsing, L.: Effect of elevated renal venous pressure on intrarenal haemodynamics. Acta Physiol. Hung., 1974, 45, 173-180.

3. Knoll, J., Makleit, S., Friedmann, T., Harsing, L. G., Jr., Hadhazy, P.: Circulatory, respiratory and antitussive effects of azidomorphine and related substances. Arch. Internat. Pharmacodyn., 1974, 210, 241-249.

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7. Knoll, J., Harsing, L. G., Jr., Friedmann, T.: Azidoethylmorphine, a new potent non-narcotic oral antitussive. Acta Physiol. Hung., 1977, 50, 341-356.

8. Vizi, E. S., Ronai, A. Z., Harsing, L. G., Jr., Knoll, J.: Inhibitory effect of dopamine on acetylcholine release from caudate nucleus. Pol. J. Pharmacol. Pharmac., 1977, 29, 201-211.

9. Vizi, E. S., Harsing, L. G., Jr., Knoll, J.: Presynaptic inhibition leading to disinhibition of acetylcholine release from interneurons of the caudate nucleus: effect of dopamine, beta-endorphin and D-Ala2-Pro5-enkephalinamide. Neuroscience, 1977, 2, 953-961.

10. Harsing, L. G., Jr., Vizi, E. S., Knoll, J.: Increase by enkephalin of acetylcholine release from striatal slices of the rat. Pol. J. Pharmacol. Pharmac., 1978, 30, 387-395.

11. Harsing, L. G., Jr., Magyar, K., Tekes, K., Vizi, E. S., Knoll, J.: Inhibition by deprenyl of dopamine uptake in rat striatum: a possible correlation between dopamine uptake and acetylcholine release inhibition. Pol. J. Pharmacol. Pharmac., 1979, 31, 297-307.

12. Harsing, L. G., Jr., Illes, P., Furst, S., Vizi, E. S., Knoll, J.: The effect of prostaglandin E1 on acetylcholine release from cat brain. Acta Physiol. Hung., 1979, 54, 177-185.

13. Magyar, K., Harsing, L. G., Jr., Tekes, K., Knoll, J.: The role of 1metabolic factors in the interaction between opiates and homopyrimidazoles in the central nervous system. Arch. Toxicol., 1980, 4, 376-379.

14. Vizi, E. S., Harsing, L. G., Jr., Zsilla, G.: Evidence of the modulatory role of serotonin in acetylcholine release from striatal interneurons. Brain Res., 1981, 212, 89-99.

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6. INVITED LECTURES

1. Dr. E. Costa, Laboratory of Preclinical Pharmacology, National Institute of Mental Health: Conversion of Arg6-Phe7-Met5-enkephalin to Met5-enkephalin in isolated organs.

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The Glutamate Receptor Ion Channels

RAYMOND DINGLEDINE,[1] KARIN BORGES, DEREK BOWIE, AND STEPHEN F. TRAYNELIS
Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia

This paper is available online at http://www.pharmrev.org

[1] Address for correspondence: Raymond Dingledine, Department of Pharmacology, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322. E-mail: rdingledine@pharm.emory.edu

I. Introduction

The ionotropic glutamate receptors are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the brain. The cloning of cDNAs encoding glutamate receptor subunits, which occurred mainly between 1989 and 1992 (Hollmann and Heinemann, 1994), stimulated this field like no other event since the recognition in the early 1980s that the *N*-methyl-D-aspartate (NMDA)[2] receptor antagonist, D-AP5, has neuroprotective and anticonvulsant properties (reviewed by Choi, 1998; Dingledine et al., 1990), and that calcium entry through glutamate receptor channels plays important roles in development and in forms of synaptic plasticity that may underlie higher order processes such as learning and memory (Maren and Baudry, 1995; Asztely and Gustafsson, 1996). These earlier findings implicated NMDA receptors in a variety of neurologic disorders that include epilepsy, ischemic brain damage, and, more speculatively, neurodegenerative disorders such as Parkinson's and Alzheimer's diseases, Huntington's chorea, and amyotrophic lateral sclerosis.

Glutamate receptors are expressed mainly in the central nervous system, but several potentially important exceptions are worth mentioning. The realization that pancreatic islet cells express glutamate receptors that modulate insulin secretion (Inagaki et al., 1995; Weaver et al., 1996, 1998) and that antagonists of NMDA receptors expressed by osteoclasts and osteoblasts slow bone resorption (Chenu et al., 1998; Patton et al., 1998) raise the possibilities that antagonists restricted to the periphery might find uses in the treatment of diabetes and osteoporosis. Moreover, there is evidence for the presence of NMDA and non-NMDA receptors in small, unmyelinated sensory nerve terminals in the skin (Ault and Hildebrand, 1993; Carlton et al., 1995). Subcutaneous injection of as little as 300 pmol of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 30 pmol of MK-801 produced analgesia for a subsequent injection of formalin into the same site. These findings raise the possibility that peripheral glutamate receptors residing on nerve terminals in the skin may be a target for certain forms of pain associated with inflammation. NMDA receptor antagonists can also reduce histamine secretion from mast cells collected from the rat peritoneal cavity (Purcell et al., 1996), and NMDA depolarizes and elevates intracellular Ca^{2+} in mouse taste receptor cells in taste buds (Hayashi et al., 1996). Numerous ionotropic glutamate receptor subunits appear to be expressed by cardiac ganglia, but their functions are unknown (Gill et al., 1998). Thus, the potential therapeutic realm of drugs targeted to glutamate receptors is expanding to include cells (neural and nonneural) in the periphery. Most recently, evidence for a role for ionotropic glutamate receptors expressed by plant cells in light signal transduction has been reported (Lam et al., 1998), suggesting that mammalian receptors may have evolved from a more primitive signaling mechanism.

The cloning of the glutamate receptors in the early 1990s has taken the study of glutamate receptor pharmacology, physiology, and pathophysiology to the molecular level. Several major reviews of the initial fruits of cloning appeared in 1994 (Hollmann and Heinemann, 1994; McBain and Mayer, 1994; Nakanishi and Masu, 1994; Gill, 1994). This review focuses primarily on the functional insights and new pharmacological targets identified by molecular biological approaches since 1994. Although synaptic functions of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have been well understood, until recently the physiological roles of kainate receptors have been elusive. The recent applications of new drugs and genetic

[2] Abbreviations: NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; UTR, untranslated region; KBP, kainate-binding protein; RT-PCR, reverse transcription-polymerase chain reaction; GABA, γ-aminobutyric acid; CNS, central nervous system; NO, nitric oxide; ABP, AMPA receptor-binding protein; AKAP, A kinase-associated protein; GKAP, guanylate kinase-associated protein; LTP, long-term potentiation; LIVBP, leucine/isoleucine/valine-binding protein; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CRIPT, cysteine-rich interactor of PDZ three; GRIP, glutamate receptor-interacting protein; EPSC, excitatory postsynaptic current; SAPAP, SAP90/PSD-95-associated protein; PSD, postsynaptic density; GK, guanylate kinase; S-SCAM, synaptic scaffolding molecule; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, synaptic NSF attachment protein; MAP, mitogen-activated protein; chapsyn, channel-associated proteins of synapse; MAP1A, microtuble-associated protein 1A; PEPA, 4-[2(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide.

knockout technology are finally providing clues to the role of kainate receptors in synaptic transmission (e.g., Clarke et al., 1997; Rodriguez-Moreno and Lerma, 1998). However, to focus this review on the properties of the receptors themselves, we do not provide detailed information on the physiological roles of the various receptors, or their regional distribution, or extensive evaluation of genetically modified mice. Several recent reviews complement this one (Edmonds et al., 1995; Bettler and Mulle, 1995; Steinhauser and Gallo, 1996; Fletcher and Lodge, 1996; Sucher et al., 1996; Ben-Ari et al., 1997; Borges and Dingledine, 1998; Dingledine and McBain, 1998; Ozawa et al., 1998; Myers et al., 1999).

II. Gene Families

The three pharmacologically defined classes of ionotropic glutamate receptor were originally named after reasonably selective agonists —NMDA, AMPA, and kainate. It turned out that NMDA, AMPA, and kainate receptor subunits are encoded by at least six gene families as defined by sequence homology: a single family for AMPA receptors, two for kainate, and three for NMDA (Table 1). Sequence similarity and, in some cases, similarity in intron-exon structure (Suchanek et al., 1995) suggests a common evolutionary origin for all of the ionotropic glutamate receptor genes. These genes are scattered over numerous chromosomes (Table 1), although the *GRIA4* and *GRIK4* genes are located near one another on the long arm of chromosome 11 and *GRIK5* and *GRIN2D* may be close together on the long arm of chromosome 19. The protein products of these two pairs of genes do not appear to interact functionally, and it is not known whether these gene pairs are coordinately regulated similar to the gene clusters of the nicotinic acetylcholine receptor (Boulter et al., 1990a).

The δ1 and δ2 genes are distant structural relatives (18–25% amino acid identity) of other glutamate receptor subunits (Lomeli et al., 1993). These orphan subunits do not form functional channels by themselves, nor have they been shown to modify the function of other subunit combinations. However, knockout of the δ2 gene leads to loss of activity-related depression of the parallel fiber-Purkinje cell synapse (Kashiwabuchi et al., 1995), and the mouse Lurcher neurological mutant has recently been shown to be caused by a gain-of-function mutation in δ2, which leads to a large constitutive inward current that may provide a genetic model for excitotoxicity (Zuo et al., 1997). The genetic knockout strategy has recently helped to delineate the potential functions of another protein distantly related to the NMDA receptor subunits, NR3A (previously named NMDA-RL). NR3A coimmunoprecipitates with NR1 and NR2B subunit proteins in homogenates of mouse cerebral cortex (but not with GluR2, GluR6, δ1, or δ2 subunit proteins). Coexpression of NR3A with NR1 and NR2A causes a reduction in both whole-cell currents (Ciabarra et al., 1995) and single-channel conductance (Das et al., 1998), and perhaps a lower Ca^{2+} permeability. Accordingly, NMDA-induced currents in cortical neurons were increased about 3-fold in NR3A knockout mice (Das et al., 1998). These findings strongly suggest that the NR3A subunit may serve a regulatory function in NMDA receptors; in particular for controlling the amplitude and Ca influx through synaptic NMDA receptor channels.

No genetic diseases in humans have yet been linked to mutations in any of the glutamate receptor subunits, although as noted above the mouse Lurcher mutant is caused by a mutation in δ2. Additionally, the genotype at a polymorphic triplet repeat in the 3′ untranslated region (UTR) of human GluR6 appears to have a minor influence on the age of onset of Huntington's disease (Rubenstein et al., 1997). A number of neurological disorders are accompanied by the appearance of antibodies to glutamate receptor subunits (e.g., to GluR3 in Ras-

TABLE 1
Glutamate receptor subunits and their genes

Group	Receptor Family	Subunit	Gene	Chromosome (human)	GenEMBL Accession No.		
					Mouse	Rat	Human
1	AMPA	GluR1	*GRIA1*	5q33	X57497	X17184	I57354
1	AMPA	GluR2	*GRIA2*	4q32-33	X57498	M85035	A46056
1	AMPA	GluR3	*GRIA3*	Xq25-26		M85036	X82068
1	AMPA	GluR4	*GRIA4*	11q22-23		M36421	U16129
2	Kainate	GluR5	*GRIK1*	21q21.1-22.1	X66118	M83560	U16125
2	Kainate	GluR6	*GRIK2*	6q16.3-q21	D10054	Z11715	U16126
2	Kainate	GluR7	*GRIK3*	1p34-p33		M83552	U16127
3	Kainate	KA-1	*GRIK4*	11q22.3		X59996	S67803[a]
3	Kainate	KA-2	*GRIK5*	19q13.2	D10011	Z11581	S40369
4	NMDA	NR1	*GRIN1*	9q34.3	D10028	X63255	X58633
5	NMDA	NR2A	*GRIN2A*	16p13.2	D10217	D13211	U09002
5	NMDA	NR2B	*GRIN2B*	12p12	D10651	M91562	U23861[a]
5	NMDA	NR2C	*GRIN2C*	17q24-q25	D10694	D13212	
5	NMDA	NR2D	*GRIN2D*	19q13.1qter	D12822	D13214	U77783
6	NMDA	NR3A	*GRIN3A*[b]			L34938	
7	Orphan	δ1	*GRID1*		D10171	Z17238	
7	Orphan	δ2	*GRID2*	4q22	D13266	Z17239	

[a] Partial sequence.
[b] Proposed here.

mussen's encephalitis—Rogers et al., 1994; Twyman et al., 1995; Carlson et al., 1997; to GluR2 in nonfamilial olivopontocerebellar degeneration—Gähring et al., 1997; and to several AMPA and kainate receptor subunits in paraneoplastic neurodegenerative syndrome—Gähring et al., 1995), but the role of these antibodies in disease manifestation is unclear (e.g., He et al., 1998).

In addition to these mammalian genes, cDNAs encoding several kainate-binding proteins (KBPs) had been isolated in the early 1990s from frog, chick, and goldfish brain that exhibit weak sequence homology to the mammalian glutamate receptors (for review, Henley, 1994). These proteins have not been found in mammals and do not seem to form functional homo- or heteromeric complexes with other glutamate receptor channels. However, chimeric proteins consisting of the channel-forming domains of KBPs and the ligand recognition domains of GluR1 or GluR6 form functional ion channels (Villmann et al., 1997), suggesting that an undiscovered modulatory subunit may be required to form fully functional KBPs.

III. Receptor Structure

A. Transmembrane Topology

The mechanism by which a receptor protein is threaded through the membrane during synthesis determines which segments face the extracellular and cytoplasmic fluids; this in turn specifies the protein domains that are available for ligand recognition, cytoplasmic modification (phosphorylation etc.), and interactions between the receptor and cytoplasmic proteins. Against initial expectations, glutamate receptors proved to have only three transmembrane domains (M1, M3, and M4) plus a cytoplasm-facing re-entrant membrane loop (M2, Fig. 1). Thus, the N terminus is located extracellularly and the C terminus intracellularly. This was deduced first by localization of endogenous and introduced *N*-glycosylation sites in KBPs (Wo and Oswald, 1994, 1995), GluR1 (Hollmann et al., 1994), GluR3 (Bennett and Dingledine, 1995), and NR1 (Wood et al., 1995), and additionally by analysis of the protease sensitivity of a reporter group fused at different positions to GluR3 (Bennett and Dingledine, 1995). The M2 segment in NMDA receptors is also thought to be a re-entrant loop based on the pattern of accessibility from both sides of the membrane of charged sulfhydryl reagents to cysteines substituted for M2 residues (Kuner et al., 1996), and the same method supports three rather than four transmembrane segments in AMPA receptors (Kuner et al., 1997). The transmembrane topology of glutamate receptors thus appears different from the four-transmembrane model of nicotinic acetylcholine receptors, but similar to that of potassium channels in that a re-entrant loop is present. Residues in this re-entrant second membrane loop control key permeation properties of the ion channel (see below).

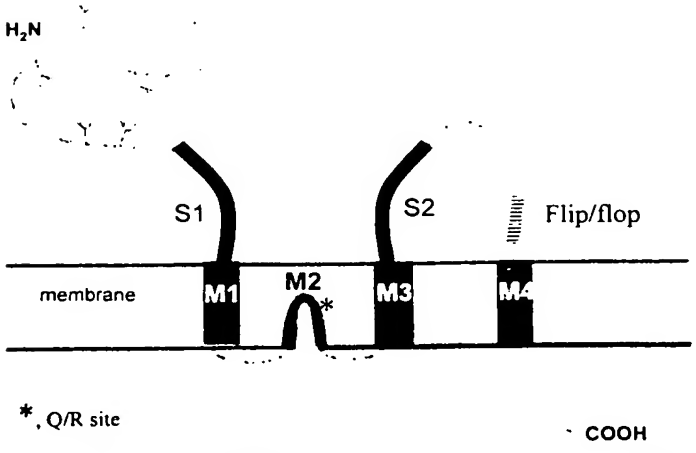

FIG 1. Structure of AMPA receptor subunits. The transmembrane topology is shown along with the flip/flop alternatively spliced exon, and the two ligand-binding domains (S1 and S2). Glycosylation sites are shown as trees in the N-terminal region.

B. Subunit Stoichiometry

Early evidence favored a pentameric structure for glutamate receptors based on the size of chemically cross-linked NMDA receptor protein (Brose et al., 1993) or functional analysis of mixtures of native and mutant subunits with different sensitivity to channel blockers (Ferrer-Monteil and Montal, 1996), although velocity sedimentation analysis was consistent with a smaller protein (Blackstone et al., 1992; Wu and Chang, 1994). Premkumar and Auerbach (1997) inferred a pentameric stoichiometry for NMDA receptors consisting of three NR1 and two NR2 subunits. Their conclusion depended on interpretation of the patterns of single-channel conductances observed in mixtures of native and mutant subunits; the asparagine residue in the M2 segment lining the channel (N616) was changed to a glutamine in the mutant. For example, coexpression in *Xenopus* oocytes of NR1(N) with a combination of NR2B (N) and NR2B(Q) produced receptors with three patterns of main and subconductance states, corresponding to those seen with 1N/2N, 1N/2Q, and a third pattern that was considered to reflect 1N/2Q/2N receptors. Because only three different channel types were observed, they concluded that there must be two and only two NR2B subunits in a receptor (i.e., Q/Q, Q/N, and N/N). When a mixture of NR1(N) and NR1(Q) were coexpressed with NR2B(Q), six single-channel current patterns were distinguishable, which by similar logic pointed to three NR1 subunits in a functional receptor. An analogous experimental design by Behe et al. (1995) found fewer single-channel patterns, however, and concluded there are only two copies of NR1, not three as Premkumar and Auerbach (1997) found. Behe et al. (1995) concluded that the most parsimonius model involved a tetrameric protein consisting of two NR1 and two NR2 subunits.

Three more recent studies have been interpreted to favor a tetrameric assembly of subunits similar to that of potassium channels (Doyle et al., 1998) or cyclic nucleotide-gated channels (Liu et al., 1998). Laube et al. (1998) found three components in the dose-response curve for activation of mixtures of wild-type and mutant NMDA receptor subunits [NR1(Q387K) and NR2B(E387A) are described further in Table 2] with either of the two coagonists, glutamate or glycine. Binomial fits to the three receptor populations were most consistent with two NR1 and two NR2 agonist-binding subunits per functional receptor. Binomial analysis of

TABLE 2
Residues important for agonist recognition in rodent glutamate receptors

Subunit	Mutation	GluR2 Equivalent[a]	Ligand Binding[b]	Functional Effect of Mutation
NR1[a, c]	Q387K	E402	β	14,000× ↑ in glycine EC_{50}, 13× ↑ in glutamate EC_{50}
	F390S	Y405	δ	63× ↑ in glycine EC_{50}
	Y392A	M407		12× ↑ in glycine EC_{50}
	F466H	Y450	β*	2,100× ↑ in glycine EC_{50}
	V666A	L650		13× ↑ in glycine EC_{50}
	S669G	G653	β	25× ↑ in glycine EC_{50}
NR1[d]	F735A	L727		16× ↑ in glycine EC_{50}
	F736A	D728		28× ↑ in glycine EC_{50}
NR1[e]	D481N	D447		7× ↑ in glycine EC_{50}
	K483Q	K449	β	130× ↑ in glycine EC_{50}
NR1[f]	D732E	E705	α*	3,700× ↑ in glycine EC_{50}
NR2B[g]	E387A	E402	β	240× ↑ in glutamate EC_{50}
	F390S	Y405	δ	50× ↑ in glutamate EC_{50}
	K459E	K449	β	180× ↑ in glutamate EC_{50}
	H460F	Y450	β*	9× ↑ in glutamate EC_{50}
	R493K	R485	α*	Complete loss of agonist responses
	S486A	P478	β*	43× ↑ in glutamate EC_{50}
	V660A	L650		20× ↑ in glutamate EC_{50}
	S664G	S654	α*	100× ↑ in glutamate EC_{50}
	V709A	M708		30× ↑ in glutamate EC_{50}
NR2A[h]	N463A	D447		6× ↑ in glutamate EC_{50}, no change in gly EC_{50}
	K465E	K449	β	10× ↑ in glutamate EC_{50}, no change in gly EC_{50}
	H466A	Y450	β*	220× ↑ in glutamate EC_{50}, no change in gly EC_{50}
	T665A	T649		7× ↑ in glutamate EC_{50}, no change in gly EC_{50}
	V666A	L650		12× ↑ in glutamate EC_{50}, 1.7× ↑ in gly EC_{50}
	G669A	G653	β	320× ↑ in glutamate EC_{50}, no change in gly EC_{50}
	T671A	T655	α*	1,000× ↑ in glutamate EC_{50}, no change in gly EC_{50}
GluR1[i]	E398Q	E402	β	8× less sensitive to glutamate desensitization
	Y446F	Y450	β*	4× less sensitive to glutamate desensitization
	L646A	L650		40× less sensitive to glutamate desensitization
	S650V	S654	α*	18× less sensitive to glutamate desensitization
GluR1[j]	E398K	E402	β	100,000× ↑ in glutamate EC_{50}
(mouse)	D443K	D447		5× ↑ in glutamate EC_{50}
	K445E	K449	β	22× ↑ in glutamate EC_{50}
GluR1[k]	K445Q	K449	β	51× ↑ in AMPA EC_{50}, 3× ↑ in glutamate EC_{50}
GluR2[k]	K449E	K449	β	4× ↑ in glutamate EC_{50}
GluR3[l]	L507Y	L483		Abolishes glutamate desensitization
	T504A	T480	α*	134× ↑ glutamate EC_{50}
GluR6[m]	N721S	T686	β	2–3× ↑ glutamate affinity, >16× ↑ AMPA affinity
cKBP[n]	E33V	E402	β	110× ↓ in kainate affinity, no change in glu affinity
	Y36F	Y405	δ	30× ↓ glutamate affinity, 5× ↓ kainate affinity
	Y73I	Y450	β*	90× ↓ glutamate affinity, 10× ↓ kainate affinity
	P100A	P478	α*	No significant change in kainate binding
	T102A	T480	α*	100× ↓ glutamate affinity, 58× ↓ kainate affinity
	R107S	R485	α*	Complete loss of kainate binding
	S266A	G653	β	5–6× ↓ kainate affinity
	S267A	S654	α*	5–6× ↓ kainate affinity
	T268A	T655	α*	Complete loss of kainate binding
	Y299A	T686	β	5–6× ↓ kainate affinity
	E316Q	E705	α*	Complete loss of kainate binding

[a] The designated amino acid residues were mapped onto the rat GluR2 sequence numbered according to Armstrong et al. (1998).
[b] Residues identified in the crystal structure of the ligand-binding domain of GluR2. *, ligand-binding residues. α, residues interacting with all agonists; β, residues predicted to interact only with specific ligands; δ, residues that maintain the shape of the ligand-binding pocket but do not contact ligands. From Armstrong et al. (1998).
[c] Kuryatov et al., 1994 (EC_{50} measured in oocytes expressing NR1/NR2B receptors).
[d] Hirai et al., 1996 (EC_{50} measured in oocytes expressing NR1/NR2B receptors).
[e] Wafford et al., 1995 (EC_{50} measured from oocytes expressing NR1/NR2A receptors).
[f] Williams et al., 1996 (EC_{50} measured in oocytes expressing NR1/NR2B receptors).
[g] Laube et al., 1997 (EC_{50} measured in oocytes expressing NR1/NR2B receptors).
[h] Anson et al., 1998 (measured in oocytes expressing NR1/NR2A receptors).
[i] Mano et al., 1996 (EC_{50} measured in oocytes expressing homomeric GluR1 receptors).
[j] Uchino et al., 1992 (EC_{50} measured in oocytes expressing GluR1 receptors).
[k] Li et al., 1995 (EC_{50} measured in oocytes expressing GluR1 or GluR1/GluR2 receptors).
[l] Stern-Bach et al., 1998 (EC_{50} measured in outside-out patches of HEK293 cells expressing GluR3 receptors).
[m] Swanson et al., 1997b (EC_{50} measured in oocytes expressing homomeric GluR6 receptors).
[n] Paas et al., 1998 (affinity judged by displacement of radiolabeled kainate from HEK 293 membranes).

the functional effect of incorporating increasing amounts of dominant negative NR1 or NR2 subunits into the receptor also supported a tetrameric assembly. Mano and Teichberg (1998) used a similar strategy to conclude that homomeric GluR1 receptors could be tetrameric complexes. Rosenmund et al. (1998) observed that upon agonist binding, activation of single receptor/channels proceeds through a staircase of openings to three different conductance levels of increasing amplitude. To resolve rapid transitions that occur during normal activation, agonist-binding sites were presaturated with the competitive antagonist, NBQX, before agonist application, so that each agonist-binding site was only made available after an antagonist molecule dissociated from the receptor. The authors proposed a model whereby the dissociation of two antagonist molecules and their replacement by two agonist molecules occurred before the first current step appeared. Current levels that were observed subsequently reflected the binding of single agonist molecules to the receptor, suggesting that each receptor contains four functional antagonist/agonist-binding sites, which is consistent with a tetrameric protein.

Thus, the conclusions of six carefully performed functional evaluations of receptor subunit mixtures (Ferrer-Monteil and Montal, 1996; Behe et al., 1995; Premkuhar and Auerbach, 1997; Laube et al. 1998; Mano and Teichberg, 1998; Rosenmund et al., 1998) are exactly split between a tetramer and a pentamer. In attempting to resolve the difference, several issues should be considered. First, each of the studies described above was designed to identify the number of functional binding sites in a receptor and relied on the assumption that each binding site in a receptor behaves independently of the others. It is well known that the agonist-binding sites of at least one other receptor, the muscle nicotinic acetylcholine receptor, show cooperativity, and negative cooperativity between binding of glutamate and glycine to the NMDA receptor is also well established (reviewed by McBain and Mayer, 1994). Second, the data of Rosenmund et al. (1998) suggest that subconductance states of a channel may be related to the number of agonists bound to the receptor. If this is correct, the interpretation of dose-response curves (Laube et al., 1998) and patterns of main and subconductance states (Behe et al., 1995; Premkuhar and Auerbach, 1997) become more complicated. Finally, post-translational processing could increase subunit complexity and potentially lead to overestimation of the number of functional subunits. Rosenmund et al. (1998) and Laube et al. (1998) argue that although their data favor a tetrameric protein, the possibility of a pentameric structure could not be entirely ruled out. Although the functional results are provocative, an unequivocal determination of the number of subunits in a functional glutamate receptor awaits physical methods that probe the structure of the protein itself.

Table 1 lists at least 14 functional glutamate receptor subunits. What pairing rules determine which subunits coassemble? Is subunit stoichiometry invariant as in the case of muscle nicotinic receptors? In the early 1990s many mixing experiments were carried out to search for instances in which coexpression of subunits from one family (NMDA, AMPA, or kainate) might alter the functional properties of receptors in a different family. These attempts were uniformly unsuccessful, and it is now generally accepted that a subunit will only assemble with others within its own family. It seems that the membrane domains may dominate assembly, because chimeras of AMPA and kainate receptors do assemble if the membrane domains are all from the same subunit (Stern-Bach et al., 1994). Functional homomeric receptors can be formed within the AMPA and kainate subunit families but probably not for NMDA receptors. Functional NMDA receptors can be formed by expression of the NR1 subunit by itself in *Xenopus* oocytes but not in mammalian cell lines. However, Soloviev and Barnard (1997) showed that a glutamate receptor subunit, XenU1, is endogenously expressed at very low abundance in *Xenopus* oocytes and can assemble with mammalian NR1 to form functional NMDA receptors. Their finding could explain why expression cloning of NR1 in *Xenopus* oocytes (Moriyoshi et al., 1991) was originally possible and reinforces the current notion that NR1 must partner with one or more NR2 subunits to form functional receptors.

Little is yet known about the exact subunit composition of native glutamate receptors, but immunoprecipitation strategies have shown that NR2A and NR2B subunits can coexist together with NR1 in native NMDA receptors gently solubilized from mammalian brain by sodium deoxycholate at pH 9 (Sheng et al., 1994; Blahos and Wenthold, 1996; Luo et al., 1997; Chazot and Stephenson, 1997); multiple NR1 splice variants can also exist in a receptor assembly (Blahos and Wenthold, 1996). Likewise, NR2D can be immunoprecipitated along with NR1 and either NR2A or NR2B (Dunah et al., 1998). Wenthold et al. (1996), using similar strategies, showed that AMPA receptors immunoprecipitated from the CA1 region of rat hippocampus (primarily pyramidal cells) consisted of two major complexes represented by GluR2 plus either GluR1 or GluR3; very few solubilized receptors appeared to contain both GluR1 and GluR3, but a small fraction of solubilized receptors appeared to be homomeric GluR1. Results from other functional assays appear compatible with heteromultimeric receptors. For example, the glycine dose-response curve of NMDA receptors assembled from NR1-1e, NR2A, and NR2C could not be described as the weighted average of dose-response curves obtained from NR1-1e + NR2A and NR1-1e + NR2C receptors done separately (Wafford et al., 1993). The glycine EC_{50} for the triple subunit combination was intermediate between those for the heterodimeric combinations. All of these results argue

for at least heteroternary NMDA receptors, but do not rule out the presence of some additional binary heteromers consisting of NR1 plus a single type of NR2 subunit.

It is apparent from the studies described above that multiple subtypes exist within the AMPA and NMDA receptor families based on subunit composition. Further complicating matters, it is now clear that multiple AMPA receptor subtypes coexist within the same neuron. Thus, Zhang et al. (1995) showed that AMPA receptor channels with both low and high calcium permeability could be found within the same retinal ganglion neuron. Washburn et al. (1997) showed that polyamine spider toxins, which selectively block GluR2-lacking AMPA receptors (Iino et al., 1996; Washburn and Dingledine, 1996), removed the inwardly rectifying component of AMPA receptor currents in hippocampal interneurons, suggesting coexpression of GluR2-lacking and -containing receptors in the same cell. Likewise, spermine (Ito et al., 1996) or polyamine spider toxins (Tóth and McBain, 1998; F. Laezza, J. Doherty and R. Dingledine, unpublished) eliminate the inwardly rectifying component of evoked *synaptic* currents in hippocampal interneurons, suggesting that mosaics of GluR2-containing and -lacking receptors are present at individual postsynaptic membranes. Tóth and McBain (1998) have proposed that the expression of calcium-permeable or -impermeable AMPA receptors is determined by the origin of synaptic input onto the dendrites of the target cell. The most direct evidence for multiple AMPA receptor subtypes in a single cell is found in the fusiform cells of the rat dorsal cochlear nucleus. In these cells, postsynaptic AMPA receptors at auditory nerve synapses on basal dendrites contain GluR4 by immunohistochemistry, whereas receptors in parallel fiber synapses on apical dendrites lack GluR4 (Rubio and Wenthold, 1997). This shows directly that subsynaptic receptor targeting can be guided by the GluR4 subunit.

The studies described above indicate that more than one AMPA receptor subtype can coexist within the same neuron. AMPA receptor diversity is even more extreme, however, because it appears that subunit stoichiometry is not fixed for AMPA receptors as it is for muscle nicotinic receptors. Washburn et al. (1997) studied three GluR2-dependent permeation features of recombinant AMPA receptors composed of different subunits in a variety of ratios, and in native receptors expressed by hippocampal interneurons: rectification, Ca^{2+} permeability and sensitivity to external polyamine block. The shape of AMPA receptor current-voltage curves in individual cells could not be described by an algebraic summation of I-V curves from two populations of receptors, those containing and lacking GluR2 in some fixed but unspecified stoichiometry. Moreover, rectification was much less sensitive to the relative abundance of GluR2 than was Ca^{2+} permeability. Both of these results argue strongly that the number of GluR2 subunits in an AMPA receptor is not fixed (see also Geiger et al., 1995). Variable AMPA receptor subunit stoichiometry endows excitatory synapses with a much wider range of responses than previously imagined.

C. Ligand-Binding Sites Located in a Hinged Clamshell-like Gorge

A high-resolution crystal structure (approximately 1.9 Å) has recently been obtained for the ligand-binding domain of GluR2 complexed with the agonist kainate (Armstrong et al., 1998). This achievement followed and built upon much effort devoted to model the structure of glutamate receptor subunits, which in turn was made possible by the realization (Nakanishi et al., 1990) that glutamate receptors share weak sequence homology with a large family of bacterial amino acid-binding proteins whose structures had been solved to high resolution. A conserved amino acid-binding pocket (Oh et al., 1993, 1994; Sun et al., 1998) is proposed to exist in all glutamate receptors. This pocket would be formed from two globular domains (S1 and S2) drawn from the sequence adjacent to the M1 domain and the M3-M4 loop, respectively (Fig. 2A). In the bacterial proteins, the two lobes of the binding pocket are in a dynamic equilibrium of open and closed states; binding of ligand stabilizes the closed form of the clamshell structure. Four studies support the idea that the agonist-binding site of glutamate receptors is also a bilobular structure. First, swapping of S1 and S2 domains between GluR3 and GluR6 subunits caused the expected change in agonist pharmacology (Stern-Bach et al., 1994). Second, a soluble (nonmembrane bound) "minireceptor" consisting of these two domains from GluR4 or GluR2 joined by a hydrophilic spacer peptide was able to bind AMPA, glutamate, kainate, quisqualate, and CNQX with the expected affinities (Kuusinen et al., 1995; Arvola and Keinänen, 1996). Similarly, a soluble glycine-binding site with correct pharmacology was preserved in a fusion protein consisting of the S1 and S2 lobes of the NR1 subunit connected by a linker (Ivanovic et al., 1998). Third, inserting the S1 domain from GluR6 into GluR2 decreased the affinity for AMPA and increased kainate affinity (Tygesen et al., 1995). Finally, deletion of the N-terminal 400 amino acids, and the C-terminal 90 amino acids, from GluR6 left a membrane-bound core homomeric receptor that displayed normal [^{3}H]kainate-binding properties (Keinänen et al., 1998). These findings support the concept that individual glutamate receptor subunits, like many other proteins, are constructed in a modular fashion: a pore-forming domain similar to that of potassium channels plus two separate domains that form a ligand-binding site similar to those of the bacterial periplasmic binding proteins (Wo and Oswald, 1995; Paas, 1998). The N-terminal 400 amino acids appear to play no significant role in ligand binding but may be the locus of many modulatory functions in some (e.g., NMDA) receptors. The crystal structure of the ligand-binding domain

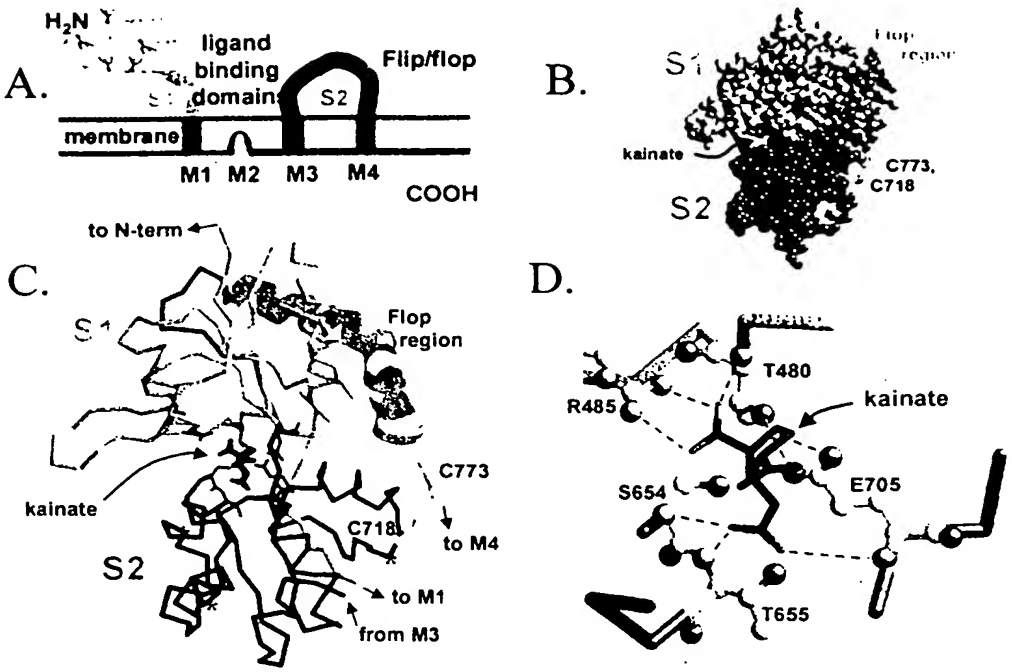

FIG. 2. Crystal structure of the GluR2 subunit and identification of agonist-binding residues. A, schematic of a glutamate receptor subunit with the two domains that contain agonist-binding residues colored in orange (S1) and turquoise (S2). The flip/flop region is indicated in violet. B, space-filled representation of the kainate-bound S1 and S2 domains joined by an 11-residue linker peptide, with coloration the same as in A. The flop domain is helical and located on a solvent-exposed face of the protein. The position of a single-kainate agonist molecule (black) within a deep gorge of the protein is indicated; the two disulfide-bonded cysteines (C718 and C773) are shown in yellow. Red asterisks mark the positions of S662 and S680 (lower left), which in GluR6 are important for PKA, and N721 (adjacent to the yellow C722), which in GluR5 and GluR6 controls agonist sensitivity. C, backbone representation of the subunit, with kainate (black) docked into its binding site. The kainate-binding residues are shown as stick figures in magenta, the two cysteines in yellow, and the flop helix structure in violet. The two green residues (E402 and T686) do not directly bind to kainate but instead interact with each other, helping to hold the clamshell in the closed conformation. D, close-up view of the ligand-binding pocket. The binding residues are in space-filled representation, with atoms colored conventionally (gray = carbon, light blue = nitrogen, red = oxygen). These images were created in rasmol from the pdb file graciously provided by E. Gouaux (Armstrong et al., 1998).

of GluR2 confirmed the bilobular structure (Fig. 2, A and B) and revealed additional details about the ligand-binding site.

Figure 2B shows a spacefill model of the GluR2 structure, with color-coded S1 and S2 lobes folding around the kainate molecule (shown partly buried in black). An immediate conclusion from inspection is that the ligand-binding pocket appears to be entirely contained within a single subunit rather than being at the interface between two subunits. The flip/flop domain (violet) is an α-helical structure on the side opposite the ligand-binding gorge. The subunit backbone is shown in Fig. 2C, where the residues that make contact with the kainate molecule are shown in magenta and kainate itself in black. The two residues in green (E402 and T686) do not participate directly in kainate binding but instead interact with one another to help hold the S1 and S2 lobes together. The two conserved cysteines that play a role in redox modulation of NMDA receptors are disulfide-bonded and shown in yellow. Figure 2D shows an enlarged view of the ligand-binding pocket, with electrostatic or polar interactions indicated by dotted lines. It is visually clear that kainate, binding deep within the S1 to S2 cleft, stabilizes the closed form of the clamshell structure by simultaneously bonding with residues in both S1 and S2 lobes.

How does ligand binding lead to channel opening? One can speculate that closure of the S1 and S2 lobes places a torque on the receptor that is transmitted to the channel region. The resulting mechanical force could increase the likelihood that the channel structure itself undergoes a conformational change to the open state. Whether desensitization is caused by a time-dependent relaxation of the "molecular spring" that connects the S1 and S2 lobes to the membrane, to partial unbinding of the agonist molecule that releases one of the lobes, to further closure of the cleft as residues in the outer reaches of the two lobes interact when the nearly closed clamshell breathes, or to some other event, remains for future work.

The ability of different agonists to either bind or activate glutamate receptors has now been assessed in well over 100 mutants in an effort to identify residues important for agonist binding. Residues that have been shown to influence agonist potency by more than a fewfold in kainate, AMPA, and NMDA receptors are identified in Table 2. These residues have been mapped onto the sequence of the GluR2 subunit so their positions can be

compared. It is immediately apparent that several amino acids appear in homologous positions in the different subunits. An example is R485, the guanidinium group of which interacts with a carboxyl group of kainate in GluR2 (Fig. 2D). This arginine is also present in NR2B and cKBP and, when mutated to a lysine or serine, abolishes agonist responses (Table 2). Other examples are found by perusing Table 2. It is remarkable that, with one exception, the correspondence is excellent between the predictions made by functional evaluation of point mutations and the direct structural identification of residues in or near the binding pocket. That exception is Pro478. In GluR2, the carbonyl group of P478 is positioned near T480 and appears to bind to the nitrogen of kainate (not shown in Fig. 2D), but mutation of the homologous proline in chick KBP had no effect on agonist binding (Table 2).

Certain residues do not participate directly in agonist recognition but instead serve allosteric roles. For example, E402 in GluR2 has its homologs in the NR1, NR2B, GluR1, and chick KBP; in all of these subunits mutation of the E402 homolog has mild-to-substantial effects on the potency of the respective agonists (Table 2). E402 does not directly contact the kainate molecule in GluR2 but instead interacts with T686 of the opposite lobe to shape the binding pocket (Fig. 2C). Other examples of important modulatory or allosteric residues are N721, which lies adjacent to the S2 lobe cysteine and regulates agonist selectivity in GluR5 and GluR6 (Swanson et al., 1998), and S662 and S680, which in GluR6 are involved in phosphorylation by protein kinase A (PKA; Raymond et al., 1993; Wang et al., 1993; Basiry et al., 1999). The positions of these residues are marked with red asterisks in Fig. 2C.

The comparison of mutagenesis and molecular modeling in Fig. 2 and Table 2 provides support for the idea that all glutamate receptor subunits have a similar folding pattern, with ligand specificities probably accounted for by differences in amino acids at key positions as argued by Paas (1998). Analysis of mutants of the NR1 and NR2 subunits led to the proposal that the glycine coagonist docking site of NMDA receptors is exclusively on the NR1 subunit, whereas glutamate binds to the NR2 subunit (Kuryatov et al., 1994; Hirai et al., 1996; Laube et al., 1997; Anson et al., 1998; see Table 2).

The combination of structure determination and functional evaluation of mutants is thus producing internally consistent views of glutamate receptor subunit structures. Not surprisingly, before the GluR2 structure had been solved, numerous investigators had constructed molecular models by aligning the S1 and S2 sequences from glutamate receptors onto the structure of the amino acid-binding proteins, and then adjusting the new structure based on energy minimization in concert with functional information provided by mutants (Stern-Bach et al., 1994; Paas et al., 1996; Sutcliffe et al., 1996; Laube et al., 1997; Swanson et al., 1997a). The general low-resolution picture provided by homology modeling is remarkably congruent across several glutamate- and glycine-binding sites of various glutamate receptor subunits. All models incorporated two hinged lobes that close upon an agonist molecule within a cleft, and this general scheme is confirmed by the GluR2 structure shown in Fig. 2. However, it is well known that identification of residues contacting ligands from electrophysiological evaluation of mutants is not straightforward, because the measured agonist EC_{50} is influenced not only by binding affinity but also by the ease with which the agonist-bound structure undergoes the conformational change leading to channel opening (e.g., Colquhoun and Sakmann, 1998). As caveats to the uncritical interpretation of mutagenesis experiments, one only needs to recall the once widely held but mistaken views that the acetylcholine-binding pocket of acetylcholinesterase consists of acidic and basic residues, and that the selectivity filter of potassium channels is derived from π-orbitals of aromatic residues. Both of these views were overturned when crystal structures became available (Harel et al., 1993; Doyle et al., 1998). Further understanding of the structure of the ligand-binding sites of glutamate receptors will require solving the structures of other subunits liganded to a variety of agonist and antagonists. This work is important to direct and interpret a myriad of mutagenesis studies and, in the long run, to facilitate the design of new drugs. The structure of the pore inferred from functional measurements is discussed below.

IV. RNA Modifications That Promote Molecular Diversity

As described elsewhere in this review, functional diversity in glutamate receptors is determined in large part by which genes are expressed in a given neuron. In addition, ionotropic glutamate receptor subunits are subject to post-transcriptional alterations—alternative splicing and RNA editing—both of which give rise to a high structural and functional diversity.

A. Alternative Splicing

All four AMPA receptor subunits occur in two alternatively spliced versions, flip and flop, that are encoded by exons 14 and 15 (in GluR2) positioned just before the M4 domain (Figs. 1–3) (Sommer et al., 1990; Monyer et al., 1991). Flip variants predominate before birth and continue to be expressed in adult rats, whereas flop variants are in low abundance before the eighth postnatal day and are up-regulated to about the same level as the flip forms in adult animals. The flip forms of most subunits desensitize more slowly and less profoundly than the flop forms (Table 4). Desensitization in the flip forms is more potently attenuated by cyclothiazide, whereas PEPA (4-[2(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide) preferentially reduces the desensitization of the flop forms (see below).

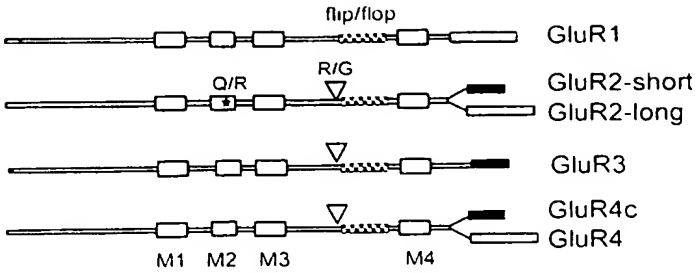

FIG. 3. Alternative splicing and editing of AMPA receptor subunits. The flip/flop and C-terminal splice variants of the AMPA receptor subunits are depicted schematically. The regions buried in the membrane are shown as boxes M1 to M4. The Q/R- and R/G-editing sites are indicated, as well as the defined phosphorylation sites in GluR1, Ser845 for PKA and Ser831 for PKC and CAMKII. Homologous C termini contain the same pattern.

C-terminal splice variants are found in GluR2, GluR4, and the kainate receptor subunits GluR5 to 7 (Figs. 3 and 4). A small percentage of GluR2 protein exhibits a long C terminus (Köhler et al., 1994). The cerebellum expresses GluR4c (Gallo et al., 1992), which has a C terminus that is shorter than that of GluR4 and is homologous to the tail of GluR2short. GluR5 cDNAs display four different C-tails and, additionally, an exon encoding 15 amino acids in the N terminus occurs in some transcripts (Sommer et al., 1992; Gregor et al., 1993). GluR6 and GluR7 each have two splice variants that differ in their C termini (Gregor et al., 1993; Schiffer et al., 1997a). When expressed as homomeric receptors in HEK 293 cells, GluR7a receptors gave rise to 5- to 10-fold larger currents than GluR7b (Schiffer et al., 1997a). Additional functional differences among the different splice variants have not been reported, but the different C termini may bind to different intracellular proteins and thus influence receptor targeting. For example, association of glutamate receptors with recently identified proteins containing PDZ domains is dependent on the C-terminal amino acids [e.g., GluR2 binding to glutamate receptor-interacting protein (GRIP), see below].

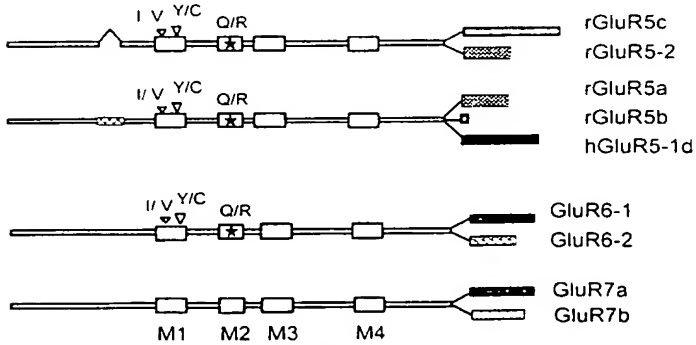

FIG. 4. Alternative splicing and editing of kainate receptor subunits. The diagram shows the basic structures of rat (prefix r) and human (prefix h) kainate receptor subunits, including the membrane-buried domains M1 to M4, the alternatively spliced cassettes, and editing sites in both M1 and M2 membrane domains.

The NR1 subunit contains three alternatively spliced exons: exon 5 in the N terminus (also called the N1 cassette) and exons 21 and 22 in the C terminus (also called C1 and C2 cassettes). Exon 22 (C2) contains an alternate acceptor splice site that, when used, splices out part of exon 22 including the stop codon and engages a new reading frame that encodes an alternative cassette C2′ before a stop codon is reached. Several nomenclatures are used for the eight NR1 splice variants, some related to structure and others referring to the chronological appearance of the clones (see Hollmann et al., 1993; Zukin and Bennett, 1995). According to Hollmann's nomenclature, NR1-1 is the full-length clone containing both C-terminal exons. NR1-2 lacks exon 21, NR1-3 lacks exon 22, and NR1-4 lacks both (Fig. 5). The lower case letters a and b indicate the presence (a) or absence (b) of exon 5. These splice variants vary considerably in their properties and are differentially localized in the adult and developing animal (e.g., Laurie and Seeburg, 1994; Laurie et al., 1995; Nash et al., 1997; Paupard et al., 1997; Weiss et al., 1998). For example, recombinant NR1 receptors lacking exon 5 (N1 cassette) have a higher affinity for NMDA, are potentiated by Zn^{2+} when expressed without NR2 subunits in *Xenopus* oocytes but are more sensitive to block by Zn^{2+} and protons when expressed with NR2 subunits, and show stronger potentiation by polyamines through relief of proton inhibition (see below; Durand et al., 1993; Hollmann et al., 1993; Traynelis et al., 1995, 1998). Receptors containing N1 without C1 and C2 are more strongly enhanced by phorbol esters than those with C1 or C2 (Durand et al., 1993). Interestingly, after optic nerve crush, retinal ganglion cells down-regulated NR1 subunits and expressed preferentially receptors lacking exon 5 before cell death (Kreutz et al., 1998). However, the remaining NR1-b subunits seem to be crucial for survival because experimental reduction of NR1-b by treatment with antisense oligonucleotides increased retinal ganglion cell death after nerve crush.

The C1 cassette found in NR1-1 and NR1-3 is involved in receptor clustering, i.e., it binds to neurofilaments

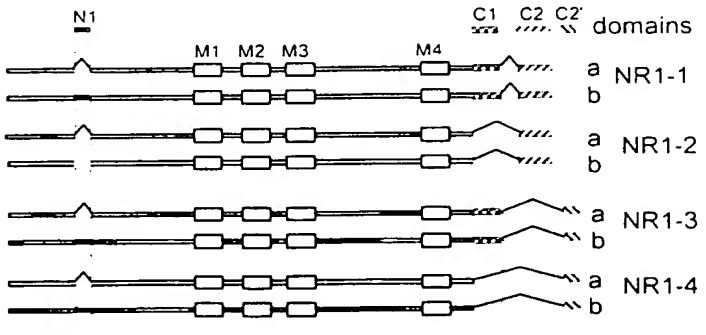

FIG. 5. Alternative splicing of NMDA receptor subunits. The different NR1 subunit splice variants arise from alternative splicing of the exons 5, 21, and 22, giving rise to the cassettes N1, C1, C2, and C2′.

and the intracellular protein yotioa (Ehlers et al., 1998; Lin et al., 1998). Furthermore, the C1 cassette contains protein kinase C (PKC) phosphorylation sites and binds to calmodulin. Clustering and interaction with these regulators can be inhibited by PKC phosphorylation in the C1 cassette (Ehlers et al., 1995). After kindling a transient reduction of C1-containing splice variants was found in rats (Kraus et al., 1996; Vezzani et al., 1995), but whether the expected functional receptor alterations contribute to the kindled state is unknown. NR1 variants with the C2′′cassette (NR1-3 and -4) interact with postsynaptic density (PSD)-95 proteins (see below).

B. Editing of AMPA and Kainate Receptors

Some glutamate receptor RNAs are post-transcriptionally modified by RNA editing, which leads to single-amino acid exchanges (reviewed by Seeburg, 1996). In this process, selected adenosines are deaminated to inosines by dsRNA adenosine desaminases (Rueter et al., 1995; see below). Inosines base pair like guanosines, which changes the amino acid codon. To date, editing has not been demonstrated for any NMDA receptor RNA, but AMPA and kainate receptor RNAs are edited at multiple positions.

In the primary transcript of GluR2, GluR5, and GluR6, a glutamine codon in the M2 domain (CAG) can be edited to an arginine (CIG) at the Q/R site (Figs. 1, 3, and 4). The arginine in edited versions of GluR2 causes low calcium permeability (Hume et al., 1991), low single-channel conductance (Swanson et al., 1996), and an approximately linear current-voltage relation even in heteromeric receptors (Verdoorn et al., 1991; Hume et al., 1991; Egebjerg and Heinemann, 1993; Washburn et al., 1997). Editing at the Q/R site in GluR6 also controls anion permeability (Burnashev et al., 1996). Furthermore, some of the AMPA receptor subunits, GluR2-4, are edited at the R/G site, which is located just before the flip/flop exons (Fig. 3). The glycine codon (IGA) replacing the genomically encoded arginine (AGA) in GluR3 and GluR4 reduces and speeds up recovery from desensitization (Lomeli et al., 1994). Finally, the kainate receptor subunits GluR5 and GluR6 can be edited in M1 at the I/V and Y/C sites (Köhler et al., 1993). Nutt et al. (1994) identified two potential editing sites in human GluR7 that are not the result of adenosine deaminations, based on sequencing reverse transcription-polymerase chain reaction (RT-PCR) products of fetal and adult human brain, which revealed different codons in the amino-terminal region of GluR7: Ser310 (TCC) or Ala (GCC) and Arg352 (CGG) or Gln (CAG). Whether these changes are due to editing as originally proposed or to polymorphisms (Schiffer et al., 1997b) awaits resolution. Since in GluR6 the edited amino acids in M1 can influence the ion permeability in receptors that have a Q at the M2 Q/R site, it has been argued that M1 may influence the structure of the open channel (Köhler et al., 1993; Burnashev et al., 1995, 1996). Changing the homologous amino acids in M1 of recombinant GluR4 had no effect on ion permeability, implying subtle differences in the channel structure between AMPA and kainate receptors. In single-cultured hippocampal neurons, editing of GluR5 and GluR6 occurred at different levels at all three sites, producing eight different species of mRNA (Ruano et al., 1995).

The enzymology of editing has received a lot of attention, with the ultimate aim of manipulating the editing process itself. Editing of glutamate receptors was initially found to depend on intronic sequences containing an editing complementary sequence, which base pairs with the exonic sequences (Higuchi et al., 1993; Egebjerg et al., 1994; Herb et al., 1996). The dsRNA structure is recognized by one of two editing enzymes, the first called DRADA or dsRAD (recently renamed ADAR1) and the second called RED1 or DRADA2 (now ADAR2) (Bass et al., 1997). Recombinant RED1 edits the GluR2 Q/R site (Melcher et al., 1996; Lai et al., 1997a; O'Connell et al., 1997), whereas the Q/R site of GluR6 can be edited by recombinant DRADA (Herb et al., 1996). Both recombinant enzymes can edit the R/G site. However, editing might not depend on the editing enzymes DRADA or RED1 alone, since some cells clearly express the mRNA for these enzymes but have no editing activity (Lai et al., 1997b). Moreover, the site selectivity and the efficiency of recombinant DRADA is changed by incubation with nuclear extracts (Dabiri et al., 1996), suggesting the contribution of additional proteins in the editing process. In addition to these deaminations that result in changes of the amino acid sequence, other adenosines are edited in introns and exons of GluR2 and GluR6, but do not change the coding sequence.

During development, editing occurs at different levels for all editing sites. The GluR2 Q/R site is the most vigorously edited site and this is even essential for survival. After E14 in rats >99% of GluR2 mRNA has arginine at the Q/R site. Removal of the editing complementary sequence in one **GRIA2** allele in mice, which reduced the efficiency of Q/R site editing by about 25%, resulted in epilepsy and early death, attesting to the importance of efficient GluR2 editing (Brusa et al., 1995). The physiological role, if any, of unedited GluR2(Q) is unclear, however, as mice engineered to contain a genomic arginine codon in the GluR2 Q/R site were phenotypically similar to normal mice (Kask et al., 1998). Also, in other species, GluR2 is nearly fully edited, with exceptions found in tissue from older humans (Nutt and Kamboj, 1994) and in fish, where the Q/R site arginine in GluR2 is genomically encoded (Kung et al., 1996). A lower extent of editing is found at the other editing sites, which gives rise to a high number of functional variants. During development, editing at the other sites increases gradually and, in the adult, only about 50% of GluR5 and 80% of GluR6 are edited at the Q/R site (reviewed by Seeburg, 1996). About 80 to 90% of GluR2-4 are edited at the R/G site, except for GluR4flip

(50%) (Lomeli et al., 1994). Kindling did not change editing of the Q/R site in GluR2, 5, or 6 (Kamphuis and Lopes da Silva, 1995), whereas transient ischemia in rats increased Q/R editing for GluR5 but reduced Q/R editing for GluR6 (Paschen et al., 1996). These results indicate that the editing processes are themselves tightly regulated.

V. Post-translational Modifications

Phosphorylation of ion channels is an important regulatory mechanism that may underly synaptic plasticity. The location of phosphorylation sites on glutamate receptor subunits and the functional consequences of phosphorylation have received much attention in the past 5 years. The responses of ionotropic glutamate receptors to agonists is usually potentiated after phosphorylation, but phosphorylation of NR1 can also disrupt channel clustering in transfected cells. The localization of the phosphorylation sites in glutamate receptor subunits was hotly debated for several years due partly to early uncertainties regarding receptor topology. Most of the controversies have recently been resolved and most experimentally veriified phosphorylation sites seem to be located intracellularly.

A. Phosphorylation of AMPA and Kainate Receptors

Like many other proteins, glutamate receptors are under tight control by various phosphokinases (reviewed by Roche et al., 1994; Soderling et al., 1994; Smart, 1997). Neuronal AMPA receptor activation can be potentiated by PKA (Knapp et al., 1990; Greengard et al., 1991; Wang et al., 1991; Blackstone et al., 1994), PKC (Wang et al., 1994a), calcium/calmodulin kinase II (CAMKII; McClade-McCulloh et al., 1993; Tan et al., 1994), and other unspecified kinases (e.g., Nakazawa et al., 1995). The potentiation by PKA of native AMPA receptors in cultured neurons appears to be due to an increase in channel open probability (Knapp et al., 1990) or open time (Greengard et al., 1991). Ser845 of GluR1 is a probable PKA target, because the C-terminus of the GluR1(S845A) mutant could not be phosphorylated after incubation with PKA (Roche et al., 1996). Moreover, the Western blot signal of hippocampal slices with antibodies directed againstphosphorylated Ser845 increased after forskolin treatment (Mammen et al., 1997). PKA activation appears to increase the open probability in recombinant GluR1 receptors but not in GluR1 (S845A) receptors (Banke and Traynelis, 1998). Changes in the phosphorylation state of Ser845 is specifically associated with synaptic plasticity. During chemically induced long-term depression, a decrease of phosphorylated Ser845, but not phosphorylated Ser831, was found (Lee et al., 1998). On the other hand, 15 min after a kindling stimulus phosphorylation of Ser845, but not Ser831, was specifically increased by about 25% (Wang et al., 1998). This suggests a role for the GluR1 PKA phosphorylation site in synaptic depression and enhancement. In Purkinje cells, according to immunocytochemistry with antibodies directed against Ser696 of GluR2, phosphorylation is suggested to occur between the M3 and M4 segments, which is a proposed extracellular domain (Nakazawa et al., 1995). Exposure to AMPA increased immunostaining, but the kinases responsible have not been identified yet. However, several early studies of recombinant or native receptors did not find AMPA receptor phosphorylation by PKA in this region (Tan et al., 1994; Moss et al., 1993), leading to the suggestion that the association of PKA with A kinase-associated proteins (AKAPs) may in some cases be required for potentiation of AMPA receptors (Rosenmund et al., 1994).

GluR1 and GluR2/3 in the postsynaptic densities could be phosphorylated by endogenous kinases in the presence of calcium and calmodulin (Hayashi et al., 1997). Phosphorylation of AMPA receptors by CAMKII and possibly PKC was produced by electrical stimulation patterns that induced long-term potentiation (LTP). Phosphorylation by CAMKII correlated temporally with the increased AMPA receptor-mediated responses during LTP (Barria et al., 1997a). The idea that phosphorylation of AMPA receptors by CAMKII mightcontribute to synaptic plasticity is supported by the finding that mice engineered to lack the α subunit of CAMKII are devoid of LTP and short-term potentiation as well as long-term depression of synaptic transmission (reviewed by Soderling, 1996). Benke et al. (1998) used nonstationary noise analysis to find evidence for an increased single-channel conductance of synaptic AMPA receptors after LTP, but it is not known whether receptor phosphorylation is involved.

The CAMKII and most PKC phosphorylation sites were initially proposed to be located between M3 and M4, which would place them on the extracellular side of the membrane. However, later studies showed that Ser627, which was believed to be involved in the potentiation by CAMKII, is not phosphorylated (Yakel et al., 1995; Roche et al., 1996). Ser831 in the C terminus of GluR1 was recently identified as the CAMKII phosphorylation acceptor site using mutagenesis and antibodies directed againstphosphorylated peptides (Roche et al., 1996; Barria et al., 1997b; Mammen et al., 1997). This serine is also the target of PKC and is unique in GluR1, not being found in GluR2-4. In hippocampal slices, Ser831 and also the other PKA target Ser845, were found to be phosphorylated under basal conditions by immunoblotting (Mammen et al., 1997). Barria et al. (1997b) noted that Ser831 is not a consensus site for either PKC or CAMKII and is only poorly phosphorylated by CAMKII after LTP induction. The fact that Ser831 is not a good substrate might be a physiological checkpoint ensuring that only strong synaptic input would lead to phosphorylation and potentiation of postsynaptic currents.

PKA has also been shown to phosphorylate recombinant GluR6 homomeric receptors (Raymond et al.,

1993), and this phosphorylation of the GluR6 protein has been suggested to underlie an enhancement of whole-cell current responses (Raymond et al., 1993; Wang et al., 1993) similar to that observed with GluR1. These two studies have utilized site-directed mutagenesis to identify extracellularly localized serine residues (S684A and S666A) that are important for control of the PKA potentiation of GluR6, but it seems unlikely that these presumably extracellular serines themselves are phosphorylated (Basiry et al., 1999). Serines in the homologous position of GluR2 (S662 and S680) are located distant to the binding pocket in the crystal structure (Armstrong et al., 1998; red asterisks in Fig. 2C). The mechanism underlying the PKA-induced potentiation of GluR6, like that of GluR1, appears to be an increase in the open probability without any apparent change in response time course. Interestingly, calcineurin, a serine/threonine phosphatase that is colocalized with PKA, has the opposite effect, decreasing open probability (Traynelis and Wahl, 1997).

The effects of tyrosine kinases on non-NMDA glutamate receptors have received little study. Cotransfection of GluR1 and v-src resulted in phosphorylation of GluR1 (Moss et al., 1993), but this experiment could not distinguish between a direct or indirect effect of v-src. In synaptic membranes no phosphorylated tyrosines could be identified on GluR1 to GluR4, GluR6, GluR7, or KA2 (Lau and Huganir, 1995).

B. Serine/Threonine Phosphorylation of NMDA Receptors

NMDA receptors can be phosphorylated by PKA, PKC, and CAMKII, and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin inhibits NMDA receptor function (Lieberman and Mody, 1994). In the brain, between 10 and 70% of NR1 and NR2 subunits seem to be phosphorylated at one or more sites by PKA or PKC. This variable proportion of phosphorylated subunits should substantially increase molecular and functional heterogeneity in the NMDA receptor family (Leonard and Hell, 1997).

PKC activation has been shown to enhance NMDA receptor function in different neuronal preparations. Activation of μ opioid receptors (Chen and Huang, 1992), the protease-activated receptor PAR1 (Gingrich et al., 1997), phosphoinositol-coupled metabotropic glutamate receptors (Aniksztejn et al., 1992), and muscarinic acetylcholine receptors (Markram and Segal, 1990; Dildy-Mayfield and Harris, 1994) all potentiated neuronal or recombinant NMDA receptors presumably via activation of PKC. Phosphorylation by PKC increases the opening probability and decreases the affinity for extracellular Mg^{2+} (Chen and Huang, 1992), but the mechanisms underlying these effects are unknown. Activation of PAR1 in hippocampal neurons by thrombin or an agonist peptide can also potentiate NMDA receptor responses in a Mg^{2+}- and voltage-dependent manner that is reminiscent of PKC-induced relief of external Mg^{2+} blockade (Gingrich and Traynelis, 1998). Tingley et al. (1997) identified Ser890, Ser896, and Thr879 in the C1 cassette of the NR1 subunit as PKC targets by using antibodies directed against phosphorylated peptides. However, the C1 cassette does not seem to be responsible for PKC-induced potentiation, because recombinant homomeric NR1 receptors lacking the C1 cassette (NR1-2a) show even higher potentiation by PKC than receptors containing C1 (Durand et al., 1993). Some phosphorylation sites responsible for the Mg^{2+}-independent PKC-induced potentiation in NR1 subunits lacking C1 (Durand et al., 1993; Wagner and Leonard, 1996) might lie within NR1 but outside of C1, or might instead reside in the *Xenopus* XenC1 subunit (Soloviev and Barnard, 1997). However, this Mg^{2+}-independent potentiation also requires the carboxyl terminus of the NR2 subunit, suggesting it may be a property of the NMDA receptor channel region. Thus, receptors containing NR2C and NR2D are insensitive to phorbol ester-induced enhancement of function in *Xenopus* oocytes, whereas receptors containing NR2A and NR2B are potentiated (Mori et al., 1992). Alternatively, phosphorylation of sites in C1 may actually cause inhibition and the site(s) for potentiation may be on the NR2 subunit. Receptors without C1 would then be potentiated to a larger degree than receptors with C1. Additional work is needed to resolve these possibilities.

The large potentiation by PKC in C1-lacking homomeric NR1 receptors might also be explained partially by the observation that without C1 there is no receptor clustering. Indeed, PKC activation and phosphorylation of NR1 at Ser890 within the C1 cassette can inhibit clustering of NR1 (Ehlers et al., 1995; Tingley et al., 1997). This might also explain why in some studies PKC reduced NMDA receptor-mediated currents (e.g., Markram and Segal, 1992). Of great interest is the observation that calcium influx through NMDA receptors appears to amplify the potentiation by PKC, as shown by Zheng and Sigworth (1997) who compared wild-type NMDA receptors with receptors carrying NR1 mutations that reduce calcium permeability.

Little is known about regulation of NMDA receptors by CAMKII or PKA. The substrate for CAMKII phosphorylation appears to be Ser1303 in NR2B and perhaps the homologous serine in NR2A. This site was found to be phosphorylated in hippocampal neurons, but its function is so far unknown (Omkumar et al., 1996). A PKA phosphorylation site resides in the C1 cassette of NR1 (Ser879; Tingley et al., 1997), but the physiological changes in NMDA receptor function observed after PKA activation seem to be indirect. In hippocampal neurons, PKA activation by β adrenergic receptors potentiated NMDA receptor activity apparently by inhibiting the phosphatase calcineurin (Raman et al., 1996). In contrast, the phosphorylation of NR1 by PKA or PKC can antagonize its interaction with spectrin in vitro and

might have direct functional consequences (Wechsler and Teichberg, 1998).

The activation of NMDA receptors can be inhibited by the serine and threonine phosphatases 1, 2A, or 2B (calcineurin). In acutely dissociated dentate gyrus granule cells, calcineurin can be activated by calcium entry through NMDA receptors and shorten the open time of NMDA receptors (Lieberman and Mody, 1994). Phosphatases 1 and 2A reduced the opening probability of NMDA receptors in cultured hippocampal neurons (Wang et al., 1994b). By second-to-second adjustment of the activity of kinases and phosphatases, the responsiveness of NMDA receptors to stimuli can thus be fine-tuned.

C. Tyrosine Phosphorylation of NMDA Receptors

Several studies showed that activation of tyrosine kinases increases NMDA receptor-mediated responses in neurons (reviewed by Wang and Salter, 1994; Köhr and Seeburg, 1996; Gurd, 1997; Lu et al., 1998; Zheng et al., 1998). Glutamate-activated currents in HEK 293 cells transfected with NR1/NR2A could be potentiated by including Src or Fyn kinases in the patch pipette (Köhr and Seeburg, 1996). Src and Fyn kinases were unable to potentiate a receptor consisting of NR1 plus a C-terminal truncation mutant of NR2A, however, suggesting that the tyrosine phosphorylation sites might lie within the C-terminal domain of NR2A (Köhr and Seeburg, 1996). Src appears to be an endogenous kinase that regulates NMDA receptors, because 1) an anti-Src antibody applied to the cytoplamic surface of spinal dorsal horn neurons reduced the open probability of NMDA receptors; 2) application to the cytoplasmic surface of a high-affinity peptide that activates Src [EPQ(pY)EEIPIA] increased channel activity in inside-out patches whereas the unphosphorylated peptide, which does not activate Src, was ineffective; and 3) anti-Src antibodies coimmunoprecipitated NR1 from synaptic membranes (Yu et al., 1997). These results taken together suggest that Src may be a regulatory component of the subsynaptic protein complex that contains NMDA receptors.

How does Src kinase potentiate NMDA receptor activation? Zheng et al. (1998) found that Src potentiation of NR1/NR2A receptors could be prevented by a trace concentration (10 μM) of EDTA or other divalent chelators, and also showed that intracellular application of Src altered the Zn^{2+} sensitivity of the receptor, thereby reducing tonic inhibition by ambient Zn^{2+} present as a contaminant in the solution. Three C-terminal tyrosines (Y1105, Y1267, and Y1387) that are found in NR2A were required for this effect of Src. Only Y1267 is unique for NR2A; both Y1105 and Y1387 are also found in NR2B and 2C. The effect of Src was traced to a reduction in Zn^{2+} potency for high-affinity block (IC_{50} = 90 nM) of NR1/NR2A receptors. With the likely assumption that ambient Zn^{2+} concentrations in the brain are in the range of several hundred nanomolar, Zheng et al. (1998) proposed that one of the synaptic functions of Src may be to regulate the degree of tonic inhibition of NR2A-containing NMDA receptors by extracellular Zn^{2+}. Interestingly, NR1/NR2B receptors could also be potentiated by Src, but only in the presence of higher concentrations of Zn^{2+} that approximate the low potency (IC_{50} = 3–10 μM) block of these receptors by Zn^{2+}. The low potency for Zn^{2+} inhibition of NR2B, NR2C, and NR2D (see below) may explain the failure of Köhr and Seeburg (1996) to observe potentiation of NR1 coexpressed with NR2B, NR2C, or NR2D. In oocytes with a nominally Zn^{2+}-free bath solution, not only NR2A, but also NR2B and even NR2D-heteromeric and NR1-homomeric receptors were potentiated by Src and by an endogenous kinase that was activated by insulin (Chen and Leonhard, 1996). The potentiation by Src of NR1/NR2B and NR1/NR2D was much less than that of NR1/NR2A receptors, and the potentiation by insulin may be partially due to a PKC-dependent mechanism because insulin receptors are also linked to phospholipase C.

Which NMDA receptor subunits can be phosphorylated on tyrosine residues? Moon et al. (1994) reported that the major tyrosine-phosphorylated protein in the PSD is NR2B. Tyrosines in 2 to 4% of NR2A and NR2B subunits, but not the NR1 subunit, were found to be phosphorylated in synaptic plasma membranes (Lau and Huganir, 1995). This low proportion of tyrosine-phosphorylated subunits contrasts to the high degree of NR2 subunit phosphorylation by PKA and PKC (up to 70%). The lower level of tyrosine phosphorylation of NR2A may allow for large increases in response to environmental stimuli. Indeed, an endogenous tyrosine kinase was found to increase phosphorylation of NR2A but not NR2B or NR1 in synaptic membranes by about 7-fold (Lau and Huganir, 1995). The endogenous kinases responsible for tyrosine phosphorylation of NR2B in synaptic membranes have not been fully identified, although exogenous Fyn could phosphorylate NR2A and NR2B in the PSD fraction (Suzuki and Okumara-Noji, 1995) and Src and NMDA receptors coimmunoprecipitate (Yu et al., 1997). The phosphorylation of NR2B by Fyn kinase antagonized the interaction of NR2B with spectrin in vitro (Wechsler and Teichberg, 1998), so one function for Fyn kinase may be to target NMDA receptors to the subsynaptic membrane.

Tyrosine kinase-enhanced synaptic currents through NMDA receptor channels have been proposed to play a role in LTP induction. O'Dell et al. (1991) blocked LTP induction by tyrosine kinase inhibitors in the CA1 region, and fyn knockout mice are impaired in LTP and spatial learning (Grant et al., 1992). Src activation was reported to be necessary for induction of LTP in CA1 pyramidal cells (Lu et al., 1998). Electrical stimulation that produced LTP led to Src activation; conversely, blocking Src activation by perfusion with a Src-blocking peptide, Src(40-58), inhibited induction of LTP. On the other hand, direct Src activation by another peptide

[EPQ(pY)EEIPIA] was sufficient to enhance excitatory postsynaptic potentials, raising the question of whether Src activation alone might be sufficient for LTP. The Src-induced enhancement of synaptic excitatory postsynaptic currents (EPSCs) could be blocked by NMDA receptor antagonists (Lu et al., 1998), suggesting that this effect of Src was mediated by ongoing NMDA receptor activation. Src potentiates NMDA receptor-mediated currents and, in addition, potentiates AMPA-mediated responses dependent on NMDA receptor activation and Ca^{2-} influx. Tyrosine phosphorylation of NR2B is enhanced up to 2.5-fold beginning a few minutes after LTP induction and lasting up to at least 24 h (Rosenblum et al., 1996; Rostas et al., 1996). Moreover, tyrosine phosphorylation of NR1 and NR2B but not NR2A was increased after exposure to brain-derived neurotrophic factor (Lin et al., 1998). Brain-derived neurotrophic factor can modulate LTP and enhances EPSCs in hippocampal neurons by a postsynaptic, phosphorylation-dependent mechanism. Despite these studies, however, it is not yet clear whether LTP requires tyrosine phosphorylation of either NR2A or NR2B.

From studies with genetically engineered mice, it seems that either NR2A or NR2B is necessary for LTP depending on the synapse. The impairment of LTP by reduced NR2B and NR2A expression is synapse specific for CA3 cells that express both subunits (Ito et al., 1997). In adult heteromeric $NR2B^{-/-}$ mice, LTP was reduced at the fimbria-CA3 synapse but not at the commissural-associational input. On the other hand $NR2A^{-/-}$ mice showed specifically reduced LTP at the commissural-associational-CA3 synapse but not the fimbria-CA3 synapse. Whether this effect is due to the lack of phosphorylation or reduced association with intracellular binding proteins is not known. Mice lacking the C terminus of NR2A containing the phosphorylation sites had a similar phenotype as the NR2A knockout mice, with reduced LTP in the hippocampal CA1 region and impaired contextual learning (Sprengel et al., 1998); it is therefore likely that the C terminus and possibly its phosphorylation play some role. Interestingly, phosphorylation regulates clustering and association with NMDA receptor subunits with intracellular proteins (see below).

A number of other conditions are associated with tyrosine phosphorylation of NMDA receptor subunits. Transient global ischemia in the four-vessel occlusion rat model increased NR2A tyrosine phosphorylation by up to 29-fold for at least 24 h, with a smaller increase in the NR2B subunit (Tagaki et al., 1997). Moreover, tasting an unfamiliar substance increased tyrosine phosphorylation of NR2B in the insular cortex in a dose-dependent manner by about 60%, starting within minutes and lasting up to several hours (Rosenblum et al., 1997). In contrast to LTP, NR2B tyrosine phosphorylation after experiencing a novel taste was not blocked by AP5 and so apparently does not require NMDA receptor activation.

Conversely, endogenous tyrosine phosphatases may also regulate channel opening probability, because external application of a protein tyrosine phosphatase inhibitor in inside-out patches from rat spinal neurons increased open probability (Wang et al., 1996). Thus, tyrosine kinases and phosphatases also appear to play important roles in adjusting the activation properties of NMDA receptors.

D. Glycosylation, Proteolysis, and Covalently Bound Lipids

Glycosylation influences several properties of glutamate receptors, including channel activity and modulation by lectins. AMPA receptors contain 4 to 6 *N*-glycosylation sites, kainate receptors between 8 and 10, and NMDA receptor subunits 6 to 12 (Everts et al., 1997). Unglycosylated S1-S2 domains of GluR2 were crystallized with bound kainate (Armstrong et al., 1998), suggesting that agonist recognition does not absolutely require glycosylation. Inhibition of glycosylation by tunicamycin reduced the apparent molecular mass of GluR1 and GluR2 by about 4 kDa and prevented [^{3}H]AMPA binding (Kawamoto et al., 1995a). Tunicamycin also inhibited the functional expression of kainate and AMPA-evoked currents in oocytes injected with rat brain total RNA (Musshoff et al., 1992). In contrast, in HEK 293 cells, tunicamycin reduced the maximal amplitude of kainate-induced currents mediated by recombinant GluR1flop only by about 50% (Hollmann et al., 1994). A subsequent study showed that flip and flop variants of GluR1 to GluR4 are differentially affected by tunicamycin treatment. The kainate- and glutamate-induced currents of the flop forms are decreased by tunicamycin treatment (except for GluR4flop), whereas currents through the flip variants are increased (with the exception of GluR3flip) (Everts et al., 1997). Tunicamycin occluded the potentiating effect of the lectin concanavalin A on all AMPA and kainate receptor combinations tested (Everts et al., 1997), in accord with the idea that the lectin-binding sites on AMPA receptors involve the attached sugars.

Glycosylation of the NMDA receptors is even more extensive, e.g., about 20 kDa of the 120-kDa NR1 and the 180-kDa NR2B protein, and about 10 kDa of the other NR2 subunits consists of sugar moieties (Kawamoto et al., 1995b; Laurie et al., 1997). Glycosylation is necessary for the binding of the NMDA receptor antagonist [^{3}H]dichlorokynurenate (Kawamoto et al., 1995b) and channel function. Tunicamycin nearly abolished glutamate-evoked currents in NR2A- or NR2B-containing receptors, as well as in homomeric NR1, heteromeric NR1/NR2C, and NR1/NR2D receptors (Everts et al., 1997). Tunicamycin did not reduce the total number of binding sites for NR1/NR2A receptors expressed in HEK 293 cells but did interfere with receptor function as judged by [^{3}H]MK801 binding and excitotoxicity assays (Chazot et al., 1995). These findings suggest that

glycosylation may not be necessary for subunit assembly per se but instead may instead be required for receptor function.

Recombinant GluR6 subunits could be palmitoylated in insect cells and HEK 293 cells (Pickering et al., 1995). Mutation of cysteines 827 and 840 prevented palmitoylation and reduced PKC-induced phosphorylation. The GluR1 C terminus as well as the NR1 and NR2 C termini might be targets for the calcium-dependent protease calpain (Bi et al., 1997, 1998). Calcium treatment of synaptic membranes or brain sections gave rise to a protein fragment recognized by antibodies directed against the GluR1 N terminus in Western blots. In contrast, the signal intensity obtained with antibodies directed against the GluR1 C terminus decreased. These effects were blocked by calpain inhibitors (Bi et al., 1997). Similar results were obtained with antibodies against the NR1 and NR2 C termini. Kainate treatment of cultured hippocampal slices seemed to induce calpain-mediated proteolysis of NR2 subunits, after which the antibodies also recognized 60-kDa and 52-kDa fragments, presumably from NR2 (Bi et al., 1998). Calpain-mediated proteolysis of GluR1 was observed in organotypic hippocampal slices after NMDA or glycine exposure, which induced global LTP (Gellerman et al., 1997; Musleh et al., 1997). The proteolysis product of GluRs seem to be rare or short-lived, since lower molecular mas species have not been described elsewhere to our knowledge. The serine protease thrombin also appears to cleave native and recombinant NR1 subunits, which may have relevance for pathological conditions in which significant amounts of thrombin enter the brain parenchyma (Butler and Traynelis, 1996).

VI. Receptor Activation and Desensitization

A. Agonists

Both NMDA and non-NMDA receptors are activated by the endogenous transmitter, L-glutamate, whereas the putative transmitter candidate, L-aspartate, appears to activate NMDA receptors exclusively (Patneau and Mayer, 1990); note, however, that aspartate can also activate receptors of unknown composition (Yuzaki et al., 1996). Glycine, which was first reported to potentiate NMDA receptor activation at submicromolar levels (Johnson and Ascher, 1987), was later shown to be an essential coagonist at NMDA receptors (Kleckner and Dingledine, 1988). Early reports that some NMDA receptors can be activated by glycine alone (Meguro et al., 1992; Kutsuwada et al., 1992) have not been confirmed or rebutted. One wonders whether coassembly of NR1 with the endogenous (to *Xenopus*) XenU1 subunit may help explain this result. As described above, the glycine-binding site appears to be located on the NR1 subunit, whereas the glutamate-binding pocket is on the NR2 subunit. The structural requirements of NMDA receptor agonists at both glutamate and glycine recognition sites have been adequately covered in previous reviews (McBain and Mayer, 1994; Sucher et al., 1996).

The original classification of AMPA and kainate subtypes received support from work with recombinant receptors, which provided evidence that these agonists were selective for two different glutamate receptor subclasses. However, some members of each class of receptor can be activated by both agonists. For example, homomeric and heteromeric AMPA receptors can be activated by kainate (Boutler et al., 1990a), and certain heteromeric kainate receptors comprised of either GluR5, GluR6, or GluR7 plus either KA1 or KA2 can be activated by AMPA (Herb et al., 1992; Swanson et al., 1996; Schiffer et al., 1997a). More recent efforts have been directed at identifying selective agonists of AMPA and kainate receptors. AMPA receptor agonists are found in two major chemical classes, based on the structures of AMPA itself, or of willardiine (Gill, 1994; Fletcher and Lodge, 1996; Borges and Dingledine, 1998). Many analogs of AMPA have been synthesized that exhibit potent agonist properties, among them the carboxy derivative (*RS*)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl)propionic acid (ACPA) (Wahl et al., 1996) and a phenyl derivative, (*S*)-2-amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl)propionic acid ((*S*)APPA) (Ebert et al., 1994). In the willardiine series, 5-fluorowillardiine activates native AMPA receptors in hippocampus with a 46-fold higher potency than needed to activate native kainate receptors in dorsal root ganglia neurons (Wong et al., 1994). In addition, certain human neurotoxins—domoic acid from dinoflagellates, β-*N*-methylamino-L-alanine from cycad seeds—are also powerful AMPA receptor agonists, although domoate is also a potent kainate receptor agonist at GluR5 and GluR6 but not GluR7 receptors (Schiffer et al., 1997a).

No agonists have yet been identified that exhibit pronounced selectivity for particular AMPA receptor subunit combinations. Kainate receptors, however, are more heterogeneous in their responses to agonists. (2*S*-4*R*)-4-methylglutamate (SYM 2081) shows strong selectivity for kainate receptors (100–1000-fold compared to AMPA receptors) (Brauner-Osborne et al., 1997; Wilding and Huettner, 1997; Donevan et al., 1998). Some willardiine analogs (e.g., (*S*)-5-iodowillardiine) also show strong selectivity for kainate over AMPA receptors (Wong et al., 1994; Jane et al., 1997; Swanson et al., 1998), as does the trifluoro-kainate analog DZKA (Wils et al., 1997). DZKA has been used as a photoaffinity label of the high-affinity kainate-binding region of kainate receptors (Willis et al., 1997). Interestingly, both the *tert*-butyl AMPA derivative ATPA and 5-iodowillardiine specifically activate homomeric GluR5 receptors, the latter with no detectable affinity for homomeric GluR6 or GluR7 receptors (Clarke et al., 1997; Swanson et al., 1998). This difference in agonist sensitivity was traced to a single amino acid, N721, in GluR6 and GluR7, and S721 in GluR5 (Swanson et al., 1998). N721

is adjacent to one of the conserved disulfide-bonded cysteines in glutamate receptors and lies distant to the actual binding pocket (red asterisk in Fig. 2C). This asparagine may thus serve an allosteric role in positioning or shaping the binding pocket. This same residue was previously shown to be responsible for the small responses of homomeric GluR6 receptors to AMPA (Swanson et al., 1997a; Table 2). Although homomeric GluR6 receptors were insensitive to 5-iodowillardiine, coexpression of KA-2 and GluR6 subunits resulted in a kainate receptor that was weakly activated by this agonist (Swanson et al., 1998). The phosphono-isoxazole AMPA analog, ATPO, is another drug that has weak agonist activity at GluR5 but not GluR6 receptors (Wahl et al., 1998).

B. Competitive Antagonists: New Developments

The classical competitive antagonists of the glutamate site on NMDA receptors are phosphono derivatives of short-chain (five to seven carbons) amino acids such as AP5 and AP7, whereas halogenated quinoxalinediones and kynurenic acid derivatives were the first competitive glycine site antagonists to be identified (summarized in Priestley et al., 1995). More recently, certain phthalazinedione derivatives (Parsons et al., 1997) and benzazepinedione derivatives (Guzikowski et al., 1996) were found to be highly potent, selective, and systemically active glycine site antagonists. Although the glycine-binding site is located on the NR1 subunit, the affinity for glycine and other glycine agonists depends on which NR2 subunits are present, being about 10-fold lower for receptors containing the NR2A subunit than other NR2 subunits (Kutsuwada et al., 1992; Buller et al., 1994; Priestley et al., 1995). Honer et al. (1998) described a novel glycine site antagonist that photoaffinity labels the NR1 subunit but is more potent at NR2B-containing receptors than any other NR2 subunit. This compound, a dichloro-tetrahydroquinoline-2-carboxylic acid derivative (CGP 61594), may be useful for more precise identification of residues near the glycine-binding pocket and may also lead to drugs targeted to the NR1/NR2B subtype of NMDA receptors.

The first generation competitive blockers of non-NMDA receptors, the quinoxalines and quinoxalinediones, showed poor selectivity between AMPA and kainate receptors. Over the past 5 years much effort has being expended to develop more selective competitive blockers of AMPA and kainate receptors. Two of the earliest of these are tetrazole-substituted decahydroisoquinolines, LY293558 and LY294486, which block homomeric GluR5 kainate receptors at 1 to 10 μM but are inactive on GluR6 receptors (Bleakman et al., 1996a,b; Clarke et al., 1997). In binding assays, LY294486 showed 10- to 100-fold selectivity for GluR5 over the AMPA receptor subunits (Clarke et al., 1997). Wahl et al. (1998) showed that a phosphono analog of AMPA, ATPO, is a competitive inhibitor of recombinant AMPA receptors (Schild K_i = 8 μM against GluR1 receptors), but had no effect on homomeric GluR6 or GluR6/KA2 receptors. As mentioned above, however, ATPO was a weak partial agonist at GluR5 and GluR5/KA2 receptors. Comparison of the profile of ATPO and its close structural analogs (e.g., ATPA) may point the way to the development of more potent and selective competitive AMPA receptor antagonists.

C. Noncompetitive Antagonists

Several classes of antagonist block NMDA receptors in a voltage-independent manner without causing significant reduction in agonist potency. Among these, ifenprodil and its analogs have received the most attention, and the mechanism of action of this phenylethanolamine has been eagerly sought. High-affinity block of the NMDA receptor by ifenprodil requires N-terminal residues on the NR2B subunit (Williams, 1993; Gallagher et al., 1996). Legendre and Westbrook (1991) first concluded from single-channel measurements that ifenprodil promotes transitions to a nonconducting state of the channel. More recent kinetic experiments of Kemp and colleagues (Kew et al., 1996, 1998; Fischer et al., 1997) have extended this conclusion by showing that ifenprodil stabilizes an agonist-bound state of the receptor that has low open probability. Mott et al. (1998) tied these observations together with the known allosteric block of NMDA receptors by protons (see below), and showed that ifenprodil increases the potency of ambient protons to block the NMDA receptor. By shifting the pKa for proton block of NMDA receptors to more alkaline values, ifenprodil binding causes a larger fraction of receptors to be protonated at physiological pH and, thus, inhibited. Mott et al. (1998) propose that the "low open probability" form of the receptor identified by Kew et al. (1996) is the protonated state.

Ifenprodil is neuroprotective in animal models of focal cerebral ischemia (Gotti et al., 1988). Unfortunately, ifenprodil and several of its analogs, including eliprodil and haloperidol (Lynch and Gallagher, 1996; Brimecombe et al., 1997), block certain serotonin receptors and calcium channels in addition to NMDA receptors, limiting their clinical usefulness. Several more selective derivatives of ifenprodil are being considered for clinical development, including CP101,606 (Mennitti et al., 1997), Ro 25-6981 (Fischer et al., 1997), and Ro 8-4304 (Kew et al., 1998). One indication that these compounds may be considered for is cerebral ischemia. An interesting feature of ischemic tissue is that the pH falls, sometimes to as low as 6.5 (Silver and Erecinska, 1992). Low pH increases the potency of some but not all phenylethanolamines, as determined by electrophysiological assays on recombinant receptors and neuroprotection assays of NMDA-induced toxicity in primary cortical cultures (Pahk and Williams, 1997; Mott et al., 1998). This novel mechanism of action of a potentially therapeutically useful class of compounds highlights the util-

ity of understanding, in detail, the mechanisms underlying allosteric modulation of NMDA receptor function. The mechanism of action of ifenprodil on the proton sensor suggests an approach to optimize the design of these compounds as neuroprotectants: one could search for phenylethanolamines that are inactive at physiological pH but have ifenprodil's sensitivity boost at ischemic pHs.

At intoxicating concentrations, ethanol is another noncompetitive NMDA receptor antagonist (Peoples and Weight, 1997 and references therein); trichloroethanol, the active metabolite of the sedative hypnotic chloral hydrate, also blocks NMDA receptors noncompetitively at anesthetic concentrations (Peoples and Weight, 1998). Block by ethanol appeared more potent with the NR2A or NR2B than the NR2C or NR2D subunits (Masood et al., 1994; Chu et al., 1995), but the degree of block was not affected by pH, Zn^{2+}, or the redox state of the receptor, suggesting that ethanol might act at a novel site on the NMDA receptor (Chu et al., 1995; Peoples and Weight, 1997).

Positively charged peptides can have multiple effects on NMDA receptors, best illustrated by the effects of the dynorphin peptides. In low extracellular glycine concentration (<100 nM), NMDA receptor currents are potentiated by dynorphin peptides that contain glycine residues (Zhang et al., 1997), perhaps as a result of proteolytic release of glycine from the parent peptide by the tissue? Dynorphin A(1-13) can also inhibit NMDA receptor activation in a voltage-independent manner that is noncompetitive with either NMDA or glycine (Chen et al., 1995a,b). DynA(1-13) potency is dependent on the NR2 subunit, being greatest for NR2A (Brauneis et al., 1996); potency also increases with increasing chain length, with DynA(1-32) being the most potent (Chen and Huang, 1998). Block by DynA(1-17) is unaffected by changes in pH or in the presence of Zn^{2+}, but is substantially weakened by dithiothreitol (Chen et al., 1995b). These results suggest that the dynorphin-binding site may involve the reduced form of the NMDA receptor, but no additional information is available regarding the mechanism of block. Highly basic toxins from the Conus marine snails are also potent NMDA receptors antagonists that appear to act at polyamine-sensitive sites in a noncompetitive fashion (Zhou et al., 1996). No detailed information is available regarding their mechanism.

No noncompetitive antagonists of the kainate receptors have yet been reported, but an important group of AMPA receptor antagonists are represented by the 2,3-benzodiazepines. These compounds, unlike the 1,4-benzodiazepines, have no affinity for the γ-aminobutyric acid $(GABA)_A$ receptor but block native and recombinant AMPA receptors in a noncompetitive manner (Donevan and Rogawski, 1993; Wilding and Huettner, 1995; Bleakman et al., 1996). The (−)-stereroisomer of GYKI 53655 (also known as LY300168) is the most potent of these compounds, with an $IC_{50} \sim 1$ μM on AMPA receptors expressed by rat cerebellar Purkinje neurons (Bleakman et al., 1996). GYKI 53655 has very low affinity for kainate receptors (Wilding and Huettner, 1995; Bleakman et al., 1996). AMPA receptors with a serine-to-glutamine mutation in the S/N site of the flip/flop exon were insensitive to cyclothiazide but normally sensitive to GYKI 53655 (Partin and Mayer, 1996); morover, the potency of these compounds was not affected by the flip/flop splice variant present (Johansen et al., 1995; Partin and Mayer, 1996). Both of these observations imply that the 2,3-benzodiazepine-blocking site is different from the site responsible for allosteric potentiation by cyclothiazide. As expected, GYKI 53665 noncompetitively protected cultured rat forebrain neurons against toxicity induced by exposure to AMPA (Kovacs and Szabo, 1997).

D. Uncompetitive Blockers

An uncompetitive blocker acts only on the activated receptor, not the receptor at rest. In addition to external Mg^{2+} ions and cytoplasmic polyamines (see below), a variety of other compounds are known to enter and block open glutamate receptor channels. A general feature of these blockers is that their binding site is made available once the channel is in the open state. Thus, the rate of the onset of block is use-dependent and is accelerated by increases in open channel probability. Once bound, however, the blocker can be trapped by channel closure. Recovery from the trapped blocked state is generally slow.

This trapping block mechanism has been exploited for NMDA receptors to estimate the open probability and the time required for the first opening of NMDA channels under experimental conditions typical of central synapses (Jahr, 1992; Dzubay and Jahr, 1996). It has been speculated that use-dependent blockers may be neuroprotective against acute and chronic neurological insults, such as stroke or epilepsy, by limiting the neurotoxic damage of excessive Ca^{2+} entry into cells via NMDA receptors (Lipton, 1993). Early hopes for open-channel blockers such as phencyclidine (MacDonald et al., 1991; Lerma et al., 1991) or MK-801 (Huettner and Bean, 1988; MacDonald et al., 1991; Jahr, 1992; Dzubay and Jahr, 1996) were disappointed by the appearance of neuropsychiatric and pathological side effects (Lipton, 1993). More clinically tolerable blockers such as the dissociative anesthetic, ketamine (MacDonald et al., 1991), dextromethorphan, and the related compound, dextrorphan (Netzer et al., 1993), the des-glycine metabolite of remacemide (Subramaniam et al., 1996), or amino-adamantane derivatives, such as memantine and amantadine (Bormann, 1989; Kornhuber et al., 1989; Chen and Lipton, 1997; Blanpied et al., 1997; Sobolevsky and Koshelev, 1998), may prove to be more useful combatants against neurotoxicity. The apparently favorable outlook for the use of memantine in the treatment

of neurological diseases may reflect its partial trapping in closed NMDA channels (Blanpied et al., 1997). Block produced by MK-801 or phencyclidine is difficult to reverse. In contrast, partial trapping and release of memantine from NMDA channels favor the occurrence of strong block only during sustained receptor stimulation, which might occur during brain trauma; more limited effects are expected on normal synaptic transmission (Blanpied et al., 1997). The molecular mechanisms that govern whether a blocker will be trapped, partially trapped, or escape before channel closure are not well understood. However, as suggested from a study of adamantane derivatives, only blockers that have a binding site sufficiently deep in the pore may be trapped following channel closure (Antonov and Johnson, 1996). Similarly, an understanding of the conformational transitions that NMDA receptors undergo may also account for the inability of glutamate or glycine to dissociate from their binding sites when open channels are blocked by 9-aminoacridine (Benveniste and Mayer, 1995).

For non-NMDA receptors, several polyamine amide toxins from arthropod venom that were originally identified using invertebrate preparations (Jackson and Usherwood, 1988; Jackson and Parks, 1989; Usherwood and Blagbrough, 1991) have been shown to also block open vertebrate non-NMDA receptors at nanomolar concentrations (reviewed in Bowie et al., 1999). The most frequently studied toxins or synthetic analogs have been argiotoxin (Herlitze et al., 1993; Brackley et al., 1993) and Joro spider toxin (Iino et al., 1996) from orb-web and Joro spiders respectively, or philanthotoxin (Bähring et al., 1997; Bähring and Mayer, 1998; Brackley et al., 1990, 1993) from the digger wasp *Philanthus triangulum*. Similar to cytoplasmic polyamine block, the affinity of externally applied toxins is dependent on editing at the Q/R site (Herlitze et al., 1993; Blaschke et al., 1993; Brackley et al., 1993; Washburn and Dingledine, 1996), which has also been shown to be true of adamantane derivatives (Magazanik et al., 1997). As a result, polyamine amide toxins have proved to be useful pharmacological tools in determining the subunit composition of native non-NMDA receptors in the central nervous system (CNS; Iino et al., 1996; Haverkampf et al., 1997; Tóth and McBain, 1998; Washburn et al., 1997).

E. Antagonists with Unknown Mechanism

Nitrous oxide (laughing gas) at anesthetic concentrations was recently shown to be an antagonist at NMDA receptors (Jevtovic-Todorovic et al., 1998). A half-maximal reduction of NMDA currents in voltage-clamped hippocampal neurons was produced by 40 vol% N_2O, comparable to the anesthetic concentration of 50 to 70%. Likewise, the IC_{50} for prevention of the death of arcuate neurons in adult rats injected s.c. with 100 mg/kg NMDA was 55 vol% N_2O. N_2O had no effect on AMPA receptor responses. The N_2O antagonism of NMDA receptors appeared to be mainly noncompetitive in nature, but no additional mechanistic information is available. Interestingly, another volatile anesthetic, halothane, was shown to potentiate GluR6 responses in *Xenopus* oocytes (Dildy-Mayfield et al., 1996), an action that was traced to the M4 transmembrane domain and largely to a single amino acid (G819) in GluR6 (Minami et al., 1998).

Certain agonists at metabotropic glutamate receptors can also act as antagonists of NMDA receptors (Contractor et al., 1998). The phenylglycine derivative *d*-methyl-4-carboxyphenylglycine (MCPG), at a concentration often used to block metabotropic glutamate receptors (500 μM), reduced the current responses induced in hippocampal neurons perfused with high NMDA concentrations by 63% when the glycine concentration was very low, but had no effect when glycine was also saturating. In binding studies, however, MCPG was unable to displace radioligands that label either the glutamate- or glycine- binding site, leaving its mechanism of action unclear. A number of other drugs with actions on metabotropic glutamate receptors (e.g., (*S*)-3,5-dihydroxyphenylglycine (DHPG), *trans*-azetidine-2,4-dicarboxylate (*trans*-ADA)) had agonist activity at NMDA receptors, a finding that could be completely explained by the measured level of contamination of the commercial drug preparations with glycine and/or glutamate. This observation raises a cautionary note about interpretation of the effects of mGluR compounds on long-term potentiation (Bashir and Collingridge, 1994; Breakwell et al., 1998) and excitotoxicity (Nicoletti et al., 1996).

Endogenous sulfated steroids can modulate NMDA receptor activation (Table 3), and Weaver et al. (1997) took advantage of this observation to identify a synthetic steroid that blocks NMDA receptors in cultured hippocampal neurons with an apparently mixed competitive-noncompetitive mechanism. This steroid also appeared to have neuroprotective, anticonvulsant, and analgesic effects against chronic pain.

F. Glutamate Receptor Kinetics

The kinetic properties that underlie the time course of ligand-gated channel responses are important to understand since they provide clues as to the mechanisms governing the temporal aspects of fast synaptic transmission. A considerable amount of work on native receptor kinetics has allowed the elucidation of several features of glutamate receptor signaling that contribute to the time course of the synaptic current (reviewed by Jonas and Spruston, 1994; Edmonds et al., 1995; Jones and Westbrook, 1996; Trussell and Otis, 1996; Ozawa et al., 1998). Here, we will consider recent studies on the activation and desensitization of recombinant receptors, as well as the emergence of structural ideas about desensitization and deactivation. Both recombinant AMPA and kainate receptors are rapidly activated by high concentrations of glutamate with a high probability of opening (see Table 4). AMPA receptor activation apparently

TABLE 3
Endogenous extracellular voltage-independent modulation of NMDA receptor function

Modulator	Effect	EC_{50}	Maximal Effect[a]	
Dynorphin	Inhibition	0.3 μM[b]	100%	Chen et al., 1995 a, b; Brauneis et al., 1996, Zhang et al., 1997; Chen and Huang, 1998
Osmotic pressure	Inhibition		75%	Paoletti and Ascher, 1994
Oxidizing agents	Inhibition		70%[c]	Aizenman et al., 1989, 1990, 1992; Tang and Aizenman, 1993 a,b
Protons	Inhibition	50–200 nM[d]	100%	reviewed by Traynelis, 1998
Sulfated steroids	Inhibition	150 μM[e]	100%	Park-Chung et al., 1994, 1997
Zinc	Inhibition	0.2–2 μM[f]	100–80%	Smart et al., 1994; Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998
Arachidonic acid	Potentiation	10 μM	2-fold	Miller et al., 1992; Petrou et al., 1993; Horimoto et al., 1996, Mishikawa et al., 1994; Tabuchi et al., 1997
PACAP[g]	Potentiation	~1 μM	3-fold	Liu and Madsen, 1997; Wu and Dunn, 1997
Polyamines, histamine	Potentiation	100 μM[h]	2-fold	Johnson, 1996; Williams, 1997a,b
Reducing agents	Potentiation		3-fold	Aizenman et al., 1989, 1990, 1992; Tang and Aizenman, 1993 a,b
Sulfated steroids	Potentiation	12 μM[i]	2.5-fold	Park-Chung et al., 1997

[a] Other exogenous modulators have been described: ethanol (Peoples and Weight, 1995, 1998, Masood et al., 1994, Peoples et al., 1997); ifenprodil (Legendre and Westbrook, 1991; Kew et al., 1996; Mott et al., 1998), and conanotokins (Zhou et al., 1996). Maximal inhibition (100% is full inhibition or x-fold potentiation
[b] Dynorphin A 1-32.
[c] The response of fully reduced receptors (e.g., dithiothreitol treated) is decreased by 70% by oxidizing agents
[d] Proton inhibition depends on subunit composition
[e] 3β-hydroxy-5β-pregnan-20-one sulfate.
[f] NR2A-containing receptors are much more sensitive to extracellular Zn^{2+} than receptors containing other subunits. NR2C and NR2D are much less sensitive to Zn^{2+}.
[g] Pituitary adenylyl cyclase-activating peptide.
[h] Spermine.
[i] Pregnanalone sulfate.

requires binding of two agonists (Clements et al., 1998), whereas two glutamate and two glycine molecules appear to be required for maximum activation of an NMDA receptor (Benveniste and Mayer, 1991; Clements and Westbrook, 1991). An interesting study by Liu et al. (1998) of cyclic nucleotide-gated channels with a constrained number of ligand-binding sites argues that four agonists may be needed even though the kinetic data suggest two functional sites. Although some kainate receptor (e.g., GluR6) dose-response curves have a Hill slope of unity, cooperativity at low agonist concentrations has been suggested (Heckmann et al., 1996; Schiffer et al., 1997a). Both kainate and AMPA receptors possess low-affinity binding sites for the endogenous transmitter glutamate, and thus are thought to deactivate quickly because of the brief mean boundtime of the agonist (Table 4). Rise times of these receptor responses recorded in excised patches approach the resolution of piezo-electric liquid filament exchange systems (0.2–0.4 ms), and agonist-binding rates may approach the diffusion limit.

Gating of AMPA and kainate receptors by glutamate is extremely fast in contrast to the slow gating of NMDA receptors. The EC_{50} values for the peak response to rapid glutamate application are similar for some AMPA and kainate receptors, although GluR7 receptors exhibit unusually low affinity (Table 4). Glutamate activates NMDA receptors with much higher potency (~1 μM), and the EC_{50} is controlled by subunit expression and splicing (see Table 4 and McBain and Mayer, 1994). The different affinity of NMDA and AMPA receptors for glutamate has an important functional consequence. Since AMPA and NMDA receptors are, in many cases, colocalized at central synapses, the rapid activation and brief open time of AMPA facilitates unblock of NMDA receptors by Mg^{2+} and therefore participation of the more slowly activating NMDA receptors in synaptic currents.

All AMPA and kainate receptors desensitize rapidly and profoundly in the continued presence of glutamate, and understanding the biophysical and molecular nature of this process is central to understanding the function of these receptors in synaptic transmission as well as the effect of postsynaptic glutamate receptor function on information processing (Jones and Westbrook, 1996; Trussell and Otis, 1996). For AMPA and kainate receptors, the relative contribution of the deactivation and desensitization rates to the time course of the synaptic current is determined by the time course of glutamate in the synaptic cleft (Jonas and Spruston, 1994; Clements, 1996; Westbrook, 1996). If glutamate remains in the cleft for a very brief duration (i.e., approximately 1 ms or less), the deactivation kinetics of the postsynaptic glutamate receptors will dominate the time course of the synaptic current. However, receptor desensitization will limit the duration of excitatory postsynaptic currents when the synaptic glutamate concentration remains elevated for prolonged periods, e.g., as transmitter spills over to adjacent synapses during high frequency stimulation. AMPA receptors recover from desensitization with time constants that are approximately 10-fold faster than kainate receptors (Table 4), which is the one of the main kinetic distinctions between AMPA and kainate receptors activated by the endogenous neurotransmitter glutamate.

Many compounds have been identified that can interact with AMPA receptors (aniracetam, diazoxide, cyclothiazide, PEPA, thiocyanate) and kainate receptors (concanavalin A) to reduce desensitization (Vyklicky et al., 1991; Yamada and Rothman, 1992; Bowie and Smart, 1993; Yamada and Tang, 1993; Partin et al.1993,

TABLE 4

Kinetic parameters describing glutamate activation of AMPA, kainate, and NMDA receptors

	EC_{50}[a]	Popen[b]	τ-Deactivate[c]	Open Times[d]	τ-Desensitize	τ-Recovery	SS/Peak Ratio[e]
	μM		ms	ms	ms	ms	
GluR1flip	500	0.4–1.0	0.8–1.1		2.5–4.1	147	0.014
GluR1flop			0.8–1.1		3.2–3.7	147	0.007
GluR3flip					4.8	15–36[f]	0.024
GluR3flop					1.4		
GluR4flip	560		0.6	0.14, 3.3	3.6	6–14[f]	0.006, 0.040
GluR4flop			0.6		0.9	31–43[f]	0.003
GluR1flip/GluR2[g]					3.4–5.1	28–67[f]	0.009
GluR1flop/GluR2[g]					2.8–8.1		0.004
GluR3flip/GluR2[g]					2.9–4.9	15–26	0.015–0.022
GluR4flip/GluR2[g]				0.5, 1.3	3.7–6.1		
GluR4flop/GluR2[g]					0.8–1.1		
GluR5Q	630			0.3, 0.6	4.1–9, 68.6	50, 5000	0.01
GluR6Q	500	0.5–1.0	2.5	0.6, 2.3	4.3–4.9	1900–2400	0.04–0.009
GluR7a	5900				8.4–9[h]		0.04
GluR5Q/KA2				0.3	1.4	3000	
GluR6Q/KA2				0.4, 2.1	2.3		
GluR7a/KA1					7.6		0.03
GluR7a/KA2					5.3		0.03
NR1-1a/NR2A	1.8	0.36	33–70, 247–350[f]	0.06, 1.0, 3.6	649–750	300–618, 1200	0.28
NR1-1a/NR2B	0.9		71, 538	0.6, 2, 8		1014	
NR1-1a/NR2C	1.0		260–376[f]	0.6			
NR1-1a/NR2D	0.4	0.04	45, 4408	0.10, 0.9, 2.6	NA	NA	NA

NA, not applicable; NR1/NR2D receptors show no apparent desensitization in the continued presence of agonist. AMPA receptor data from Lomeli et al. (1994), Mosbacher et al. (1994), Partin et al. 1996), Swanson et al. (1997b), Wahl et al. (1997), and Banke and Traynelis, (1998). Kainate receptor data from Sommer et al (1992), Heckmann et al. (1996), Swanson et al 1996, 1997a), Schiffer et al. (1997a), and Traynelis and Wahl (1997). NMDA receptor data from Ikeda et al. (1992), Monyer et al. (1992), Stern et al. (1992), Ishii et al. (1993), Varney et al. (1996), Krupp et al. (1998), Vicini et al. (1998), Villarroel et al. (1998), and Wyllie et al. (1998).

[a] EC_{50} values were determined for the peak response to rapid application of glutamate.

[b] Popen was determined for AMPA and kainate receptors using non-stationary variance analysis and depends on the phosphorylation state, with phosphatases like calcineurin favoring low Popen and kinases such as PKA favoring high Popen (Traynelis and Wahl, 1997; Banke and Traynelis, 1998). NMDA receptor Popen was determined from individual activations of single channels (Wyllie et al., 1998).

[c] Deactivation time constants were measured in excised membrane patches in response to a 1-ms pulse of agonist.

[d] Individual open times are shown even for receptors for which single activations clearly occur in bursts (e.g., NMDA receptors).

[e] The ratio of the steady state to peak current was determined during prolonged application of glutamate. Editing of R to G at the R/G site increases the steady-state current for GluR4flop.

[f] See Lomeli et al. (1994) for control of the recovery from desensitization to 1-ms pulses by editing at the R/G site

[g] See Mosbacher et al. 1994) for control of desensitization kinetics by GluR2 flip and flop

[h] GluR7a and GluR7b possessed similar desensitization time courses

Similar decay times were found with NR1 splice variants.

Deactivation was measured from the relaxation current following rapid removal of glutamate and glycine.

1996; Sekiguchi et al., 1997). Although the exact mechanisms of these drugs are still under investigation, the subunit and splice variant-specific manner of their actions (Partin et al., 1994; Johansen et al., 1995; Schiffer et al., 1997a) have helped to move the concept of receptor desensitization onto firmer molecular footing (see below).

In contrast to AMPA and kainate receptors, NMDA receptors activate slowly with a τ_{rise} of 10 to 50 ms (Wyllie et al., 1998) and deactivate with a much slower time course (see Table 4). Slow deactivation could reflect entry into several closed states that precede receptor activation (reviewed by Benveniste and Mayer, 1991; Lester and Jahr, 1992; Edmonds et al., 1995). Kinetic analysis of receptor activation time course suggests that the binding of four agonist molecules (two glutamate and two glycine) is required for receptor activation (Clements and Westbrook, 1991; see discussion above). The probability that agonist-bound receptors will open has been estimated indirectly using slowly reversible open channel blockers and also from single-channel measurements to range between 0.04 and 0.3 (Dzubay and Jahr, 1996; Table 4). The deactivation time course of these receptors is much longer than the time course of glutamate in most synaptic clefts (Lester et al., 1990; Clements, 1996), and thus will dictate the duration of the synaptic current. Because NMDA receptors have high affinity for glutamate, the peak synaptic glutamate concentrations are thought always to be high enough to fully activate these receptors. Although NMDA receptors desensitize in the continued presence of agonist, this desensitization is slow and complex, reflecting a variety of different processes that involve extracellular glycine, intracellular Ca^{2+} and certain intracellular proteins (see below). In summary, the kinetic properties of both NMDA and AMPA receptors seem well designed to serve their respective roles in synaptic transmission, where AMPA receptors supply a rapid depolarization in response to neurotransmitter release, which in turn can induce partial relief of the Mg^{2+} blockade of more slowly activating NMDA receptors.

G. Molecular Determinants of AMPA Receptor Deactivation and Desensitization

The rate of AMPA receptor desensitization is controlled by both subunit composition and the RNA splicing of the AMPA receptor flip/flop region (Mosbacher et al., 1994; see Table 4). The splice variants of GluR3flop

and GluR4flop desensitize 3 to 5 times faster than other homomeric receptors, and incorporation of GluR2flop also speeds up desensitization of flip variants. In addition, editing of the R/G site upstream of the flip/flop region in AMPA receptors (see above) can speed the recovery from desensitization about 2-fold, depending on the subunit composition (Lomeli et al., 1994). Partin et al. (1994) have also showed that flip/flop region determines steady-state desensitization for GluR1, which might reflect differences in their recovery rates from desensitization.

The allosteric transitions that lead to desensitization are favored by particular agonist structures: for example, AMPA but not kainate causes rapid desensitization of AMPA receptors. The structural determinants of a variety of compounds that relieve desensitization are also becoming elucidated. Both cyclothiazide and aniracetam have more pronounced effects on flip splice variants; cyclothiazide relieves desensitization almost completely on flip receptors, but only slows entry into desensitized states of flop splice variants (Partin et al., 1994; Johansen et al., 1995). The phenyoxyacetamide derivative PEPA also slows the onset of desensitization at flop splice variants (Sekiguchi et al., 1997). These unique desensitization properties of naturally occurring AMPA receptor isoforms as well as other data (Stern-Bach et al., 1994) have highlighted the region between M3 and M4 as a critical determinant of desensitization. Site-directed mutagenesis of residues in the flip/flop region has identified several residues that control the effects of cyclothiazide on AMPA receptor function. Ser750 and Asn750 appear pre-eminent among the structural determinants of GluR1flip and GluR1flop that have been investigated thus far. Conversion of Ser750 in GluR1flip to glutamine, which is the homologous residue found in the cyclothiazide-insensitive kainate receptors, abolishes cyclothiazide actions on AMPA receptors (Partin et al., 1995). A serine residue on either the GluR1 or GluR2 subunits of heteromeric receptors is sufficient to impart cyclothiazide sensitivity to the receptor (Partin et al., 1995). Similarly, introduction of a serine residue into the homologous site on GluR6 imparts some of the behavior of cyclothiazide to this modified receptor, suggesting the process of desensitization between kainate and AMPA receptor subunits is similar (Partin et al., 1995). The flip/flop domain is a helical region lying on a solvent-exposed surface of the subunit (Fig. 2, B and C). One structural model that has been proposed suggests that desensitization modulators bind directly at or near the flip/flop site, which critically interacts with other microdomains on the protein complex in a manner dependent on residue 750. Kinetic modeling of the effects of aniracetam suggest that this compound could exert its actions almost entirely through slowing of channel closing. By contrast, a more complex scheme was needed to account for the effects of cyclothiazide, with hypothesized stabilization of a nondesensitized state that could occur through a 20-fold increase in the affinity of the agonist for cyclothiazide-bound receptor (Partin et al., 1996).

Mutagenesis has also suggested additional regions that may control various aspects of desensitization such as Leu646 in GluR1 (Mano and Teichberg, 1998) and Leu507 within the S1-binding domain of GluR3 as well as Leu497 in GluR1 (Stern-Bach et al., 1998; see Table 2). Aromatic substitutions at these latter S1 residues completely relieve desensitization independent of the flip/flop region, and suggest that this residue is critically important in conformations that govern entry into and exit from the desensitized state. Consistent with functional data, the homologous leucine residue within GluR2 points away from the flip/flop helix and is not part of the ligand-binding pocket (Armstrong et al., 1998).

H. Molecular Determinants of Kainate Receptor Deactivation and Desensitization

Although less effort has been devoted to unraveling the structural basis of desensitization for kainate receptors than for AMPA receptors, several recent reports have shed light on some structural requirements for desensitization of kainate receptors. For example, concanavalin A relieves desensitization of GluR5 and GluR6 receptors but not GluR7 (Schiffer et al., 1997a), suggesting that evaluation of glycosylation sites that differ between GluR7 and GluR5/6 might identify the binding site for this lectin. Alternatively, residues between M3 and M4 might control the effectiveness of bound concanavalin A. Furthermore, the kinetic properties of the responses to a variety of receptor agonists are distinct between AMPA and kainate receptor families, and even distinct for different subunits within a receptor family (Schiffer et al., 1997a; Swanson et al., 1998). For example, kainate induces a rapidly desensitizing response at GluR6 but a slowly desensitizing response at GluR5, and the subunit-specific attributes of the temporal response of various agonists are beginning to be used as tools to dissect outstructural features underlying kinetic properties (Swanson et al., 1997b, 1998). The deactivation rate for domoate is slower for GluR6 than for GluR5, and this difference seems largely a result of Asn721 in GluR6. Exchange of this residue in GluR6 for the corresponding residue in GluR5 (Ser721) reverses the relative domoate deactivation kinetics between the two receptors and also swaps the differential AMPA sensitivity (Swanson et al., 1997b; see Table 2). This same residue also appears to control the agonist selectivity of kainate receptor subunits (Swanson et al., 1998). Similarly, mutation of Ser689 to Ala speeds the deactivation of GluR5 to domoate; conversely, GluR6(A689S) appears to slow the desensitization to kainate. Residue 689 is homologous to S654 in GluR2 (Fig. 2D; Table 2),and thus may participate in agonist recognition.

I. Molecular Determinants of NMDA Receptor Deactivation and Desensitization

NMDA receptor desensitization reflects at least three distinct processes, and thus appears more complex than the conformation changes of the receptor protein that have been interpreted as desensitized states for AMPA and kainate receptors (reviewed by Mayer et al., 1995). First, in the continued presence of glutamate, NMDA receptor responses are diminished in a time-dependent fashion that reflects negative allosteric coupling between the glutamate and glycine-binding sites. In the presence of high glutamate concentration, this form of desensitization is manifest as a decrease in glycine affinity and can be overcome with high glycine concentrations (reviewed by Mayer et al., 1989; McBain and Mayer, 1994). Second, in the presence of high concentrations of glycine, NMDA receptor responses in dialyzed small cells or excised membrane patches rapidly desensitize by 50 to 80% (Sather et al., 1990; see Table 4). The relevance of this rapid glycine-insensitive desensitization is unknown since it only appears once the intracellular constituents are well dialyzed. Third, a Ca^{2+}-dependent form of desensitization (also referred to as Ca^{2+}-dependent inactivation) has been described that requires transmembrane movement of Ca^{2+} and has been proposed to occur at an intracellular site (Clark et al., 1990; Legendre and Westbrook, 1993; Rosenmund and Westbrook, 1993; Vyklicky, 1993). The calcium-dependent desensitization is sensitive to calcium buffers used in the patch pipette and can best be observed with low EGTA as the internal calcium buffer. Regulation of the NMDA receptor by Ca^{2+} can occur when Ca^{2+} enters the cell through routes other than the NMDA receptor. The time course of this form of NMDA receptor regulation is slow (on the order of seconds), and the process is thought to be modulated by second messenger systems and to occur during synaptic transmission (Rosenmund et al., 1993; Tong et al., 1995; Raman et al., 1996). Furthermore, time-dependent increases in Ca^{2+}-dependent desensitization might be controlled by dephosphorylation by calcineurin (Tong and Jahr, 1994). Ca^{2+}-dependent changes in NMDA receptor function have been suggested to involve filamentous actin and might occur through a rearrangement of intracellular linkages between the NMDA receptor protein and intracellular scaffolding proteins that are controlled by actin, Ca^{2+}, and ATP (reviewed by Rosenmund and Westbrook, 1993; McBain and Mayer, 1994).

NMDA receptor deactivation controls EPSC time course and is dependent on subunit composition (Table 4). Interestingly, deactivation is much slower for receptors containing the NR2D subunit, which is expressed early in development (Watanabe et al., 1992; Monyer et al., 1994). Such prolonged activation might be important for the formation, stabilization, or elimination of synapses during development. Although molecular studies have not yet been reported, evaluation of the amino acid residues that control the prolonged deactivation of NR2D compared to NR2A might shed further light on regions of the receptor that couple agonist binding to receptor activation. In this section, we will focus on recent advances in our understanding of the molecular determinants of these different states of NMDA receptors.

Like deactivation, all forms of NMDA receptor desensitization are subunit-dependent. For example, Ca^{2+}-dependent desensitization is prominent for NR2A and occurs to a lesser degree in NR2D-containing receptors, but is not significant in NR2B- or NR2C-containing receptors (Medina et al., 1995; Krupp et al., 1996). Because the glycine affinity is also influenced by the NR2 subunit (Kutsuwada et al., 1992; Ishii et al., 1993), the negative coupling between glutamate and glycine binding should also be sensitive to subunit composition. In general, NR2A seems to be unique among NR2 subunits in showing fast deactivation and the most prominent calcium-dependent, glycine-dependent, and glycine-independent desensitization. The functional significance of these unique properties of NR2A has not been fully explored.

Interestingly, NR1 C-terminal deletion mutants abolish Ca^{2+}-dependent desensitization, suggesting that this region of the receptor may be involved in this process. Ehlers et al. (1996, 1998) have identified two calmodulin-binding sites in the C-terminal region of NR1 and suggest that direct high-affinity binding of calmodulin to these binding sites may be involved in the calcium-dependent desensitization of NMDA receptors. Two groups have shown that mutations that disrupt the CBS1 calmodulin-binding site on the NR1 subunit interfere with Ca^{2+}-dependent desensitization (Zhang et al., 1998; Krupp et al., 1999). The C-terminal of NR1 also binds to α-actinin 2, an actin-binding protein, and the binding of α-actinin 2 is antagonized by calcium/calmodulin (Wyszynski et al., 1997; Allison et al., 1998). Overexpression of α-actinin 2 can reduce the Ca^{2+} calmodulin-dependent desensitization of recombinant receptors, suggesting that after Ca^{2+} entry into the cell, calmodulin may compete with α-actinin 2 binding to the NR1 C-terminal (Zhang et al., 1998; Krupp et al., 1999). Ca^{2+} may also directly reduce the affinity of α-actinin for the NMDA receptor C-terminal through its interaction with the EF hands of calcium-sensitive forms of α-actinin (Krupp et al., 1999). Krupp et al. (1999) go on to show that the C-terminal peptides can decrease Popen, suggesting that association of this part of the receptor with an intracellular surface recognition site on the protein following dissociation from α-actinin 2/actin might mediate Ca^{2+}-dependent desensitization (see also Zhang et al., 1998). Thus, α-actinin 2 may provide a link between the NMDA receptors and the actin filaments (see Rosenmund and Westbrook, 1993), and its displacement from

NR1 may play a role in calcium-dependent desensitization of NMDA receptors.

NR2C and NR2D receptors do not appear to show rapid glycine- and Ca^{2+}-independent desensitization (Krupp et al., 1996; Wyllie et al., 1998). Recently, two groups have exploited this observation and used chimeric NR2A and NR2C receptors to evaluate the structural determinants of this form of desensitization (Krupp et al., 1998; Villarroel et al., 1998). Both groups identified two N-terminal domains that differentially influence glycine-independent desensitization. A four-amino acid domain just upstream of M1 region and a 190-amino acid stretch with homology to leucine/isoleucine/valine-binding protein (LIVBP) that precedes the S1 region appear to collaborate to control the degree of desensitization. Both regions have been suggested to exert important effects on desensitization, since removal of either alone is insufficient to abolish desensitization. Two residues (Ala555 and Ser556) within the pre-M1 region have been identified as critical for desensitization of NR2A receptors, and data from the AMPA receptors also suggest that this portion of the glutamate receptor subunit can influence desensitization (Stern-Bach et al., 1998). Villarroel et al. (1998) showed that exchange of these two residues in the S1 region immediately upstream of M1 (Fig. 1) of NR2A to corresponding residues (proline and alanine) in NR2C eliminates a slower component of desensitization they observed (τ = 2 s; but see Table 4), but leaves the fast component (τ = 0.3 s) intact. On the other hand, substitution of a segment of the NR2C LIVBP region into NR2A abolished the fast component of desensitization but not the slow component. Introduction of alanine and serine into these two positions in NR2C enhanced the degree and rate of desensitization (Villarroel et al., 1998), perhaps suggesting the proline residue in NR2C places a conformational constraint on protein movement that prevents access to the desensitized state. Krupp and colleagues (1998) showed the contributions of these same two regions to the glycine-independent desensitization using a C-terminal deletion mutant of NR1 which abolishes the calcium-dependent desensitization, thereby simplifying their interpretation. In their experiments, glycine-dependent desensitization proceeded with a simple exponential time course. NR2A(A555P) and NR2A(S556A) had modest effects on the time course of desensitization, but more pronounced effects on the degree of desensitization. They found that substitution of the NR2C pre-M1 region and the LIVBP region into NR2A together abolish desensitization. These data reinforce the concept of a modular design of glutamate receptors and highlight regions that link agonist-binding domains to other portions of the receptor (including pore-forming elements) as critical determinants of the coupling between ligand binding and channel gating.

VII. Endogenous Allosteric Modulators

Relatively few forms of allosteric modulation by extracellular substances have been identified for AMPA and kainate receptors, suggesting their role as mediators of fast synaptic transmission may not be amenable to graduated fine tuning by the microenvironment. That is, the role of these receptors might be to deliver a depolarization to the postsynaptic neuron that is controlled in part by gene expression. Alternatively, their insensitivity to the extracellular environment may be a way of preserving full regulation exclusively for the second messenger-linked kinases and phosphatases that control synaptic plasticity. By contrast, at least a dozen forms of allosteric modulation of NMDA receptor function by endogenous substances have been reported, which can be taken as evidence of the importance of fine-tuning NMDA receptor function. In addition, many of the allosteric modulators provide tonic inhibition under physiological conditions (e.g., Mg^{2+}, H^+, Zn^{2+}), suggesting allosteric regulation can protect against the dangers of NMDA receptor overactivation. Table 3 summarizes the voltage-independent regulation of the NMDA receptor by a host of structurally unrelated compounds and ions. Of these, we will consider recent advances in regulation of NMDA receptors by Zn^{2+}, reducing and oxidizing agents, protons, and polyamines. The reader is referred to the primary articles for other modulators of NMDA receptors listed in Table 3 as well as other review articles (Hollmann and Heinemann, 1994; McBain and Mayer, 1994).

A. Extracellular Zinc

In addition to its role in biochemistry of various cells throughout the periphery, zinc is also concentrated by certain neurons into synaptic vesicles and can be released in a Ca^{2+}-dependent manner at certain synapses such as the mossy fiber–CA3 pyramidal cell synapse. Given this potential synaptic role of Zn^{2+} in the CNS, it has attracted a great deal of attention as a possible neuromodulator of ion channels as well as a neurotoxic agent (reviewed by Smart et al., 1994; Harrison and Gibbons, 1994; Choi and Koh, 1998; Cuajungco and Lees, 1998). It has been known for some time that group IIB transition metals such as Zn^{2+} and Cd^{2+} inhibit NMDA receptors by both a voltage-dependent and voltage-independent mechanism (reviewed by Peters et al., 1987; Westbrook and Mayer, 1987; Mayer et al., 1989; Christine and Choi, 1990; Legendre and Westbrook, 1990; McBain and Mayer, 1994; Smart et al., 1994; Trombley and Shepherd, 1996). Zn^{2+} also inhibits glutamate uptake (Spiridon et al., 1998) and potentiates AMPA receptors (Mayer et al., 1989; Rassendren et al., 1990; Dreixler and Leonard, 1997), suggesting release of Zn^{2+} might favor synaptic non-NMDA receptor activation.

Recent work has shown that recombinant NMDA receptors are inhibited in a similar fashion to native receptors (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998). Voltage-dependent NMDA receptor channel block by Zn^{2+} is much weaker than for Mg^{2+} and appears to be qualitatively different, perhaps because of greater permeation of Zn^{2+} than Mg^{2+} through NMDA receptors (Mayer et al., 1989; Christine and Choi, 1990; Legendre and Westbrook, 1990; Paoletti et al., 1997). Interestingly, channel block by Zn^{2+} appears to involve some of the same pore-accessible residues as channel block by Mg^{2+} (Mori et al., 1992; Kawajiri and Dingledine, 1993; Sakurada et al., 1993; Paoletti et al., 1997). At the single-channel level, voltage-dependent Zn^{2+} block appears at low concentrations as a flickery block and at higher concentrations as a reduction in the single-channel amplitude because the individual blockages occur at frequencies beyond the recording resolution of current patch-clamp amplifiers (Christine and Choi, 1990; Legendre and Westbrook, 1990). Voltage-independent inhibition of native NMDA receptors by extracellular Zn^{2+} involves both a reduction in opening frequency and a decrease in open duration (Christine and Choi, 1990; Legendre and Westbrook, 1990).

Although heteromeric recombinant receptors containing NR1 + NR2 subunits are inhibited by Zn^{2+} in a fashion similar to neuronal receptors, NR1 subunits expressed in *Xenopus* oocytes appear to be potentiated by submicromolar concentrations of Zn^{2+} (Hollmann et al., 1993; Zheng et al., 1994), which may reflect properties of receptors comprised of NR1 coassembled with *Xenopus* glutamate receptor subunits (Soloviev et al., 1996). The voltage-independent Zn^{2+}-binding site appears to be strongly dependent on subunit composition, being influenced by the NR2 subunits as well as NR1 splice variants (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998). Particularly interesting is the finding that the receptors comprised of the NR2A subunit are much more sensitive to Zn^{2+}, being inhibited in the nanomolar range by as much as 70 to 80% (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997). Three important conclusions have been suggested from this finding. First, contaminant Zn^{2+} in experimental solutions as well as ambient Zn^{2+} present in the extracellular space could be high enough (hundreds of nanomolar) to tonically inhibit NR2A-containing NMDA receptors. This result could explain the lack of inhibition of certain neuronal receptors, which may have been fully inhibited by high ambient Zn^{2+} in control solutions (discussed in Paoletti et al., 1997). Second, a rapidly reversible form of "redox modulation" previously described for NR2A-containing receptors (Köhr et al., 1994) largely reflects chelation of contaminant Zn^{2+} from the extracellular medium by thiol-reducing reagents (Paoletti et al., 1997; Arden et al., 1998). Third, potentiation of NMDA receptors by the nonreceptor tyrosine kinase Src appears to reflect reduction in Zn^{2+} sensitivity and subsequent relief of tonic Zn^{2+} inhibition for NR2A-containing receptors (Zheng et al., 1998). Although the concentration of Zn^{2+} in the brain and particularly the synaptic cleft remains a complex question (reviewed by Smart et al., 1994), it is clear that Zn^{2+} can have a multitude of effects on NMDA receptor function. Furthermore, Zn^{2+} permeation through unblocked NMDA receptors, AMPA receptors, and depolarization-activated Ca^{2+} channels could be detrimental to neuronal survival (Weiss et al., 1993; Koh et al., 1996; Sensi et al., 1997; Ascher, 1998; Yin et al., 1998).

What is the nature of the voltage-independent Zn^{2+}-binding site on the NMDA receptor? Given the impressive effect of the NR2 subunit on the IC_{50} value for Zn^{2+}, one might expect the Zn^{2+}-binding site to reside on this subunit. Although there is currently only indirect structural data describing the role of NR2 in presumably high-affinity Zn^{2+} binding (Köhr et al., 1994), site-directed mutagenesis of the NR1 subunit has provided some interesting clues to the structural nature of the Zn^{2+}-binding site. Amino acid substitutions at a variety of acidic residues (e.g., Glu342, Asp669) as well as presumed pore-forming residues (Asn616) and cysteine residues thought to be involved in redox modulation (Cys744, Cys798; Sullivan et al., 1994) all reduce the Zn^{2+} IC_{50} (Zheng et al., 1998; Traynelis et al., 1998). Although it is tempting to speculate that these substitutions might remove one of the electron donors to the Zn^{2+} coordination site, other interpretations are possible. Most notably, the shifts in the IC_{50} values for Zn^{2+} show a strong correlation with changes that these same mutations induce in the IC_{50} value for protons (Traynelis et al., 1998). If the proton sensor were a single ionizable residue that was closely associated with structural components of the gate, then one possibility is that this residue might also participate in Zn^{2+} coordination. Thus, any perturbation of the pKa at the proton sensor (e.g., through shielding by exon 5 or inductive effects of nearby charged residues) might also perturb Zn^{2+} binding. Although more work is needed to evaluate this possibility, these data nevertheless raise the idea that the NR1 subunit might control or participate in Zn^{2+} binding. The link between proton and Zn^{2+} regulation also suggests common structural determinants for these two important forms of extracellular regulation of NMDA receptor function (Wu and Christensen, 1996; Traynelis et al., 1998).

B. Reduction and Oxidation of Extracellular Cysteine Residues

Neuronal NMDA receptor function appears to be unusually sensitive to the oxidizing potential of the extracellular environment (reviewed by Aizenman et al., 1989; McBain and Mayer, 1994; Gozlan et al., 1994; Aizenman, 1994), and this redox modulation is controlled in recombinant receptors by two cysteine resi-

dues (Cys744 and Cys798) on the NR1 subunit (Sullivan et al., 1994), as well as which the NR2 subunit is present (Köhr et al., 1994; Sullivan et al., 1994; Omerovic et al., 1995; Brimecombe et al., 1997). The location of these cysteines in the GluR2 structure is shown in yellow in Fig. 2, B and C. When these cysteines are oxidized by experimental reagents such as 5,5′-dithiobis(2-nitrobenzoic acid), the receptor response is attenuated, and when they are reduced with compounds such as dithiothreitol the receptor response is enhanced. The reduced receptor is associated with a roughly 2-fold higher single-channel opening frequency and slightly lower EC_{50} value for NMDA, but no change in single-channel conductance (Tang and Aizenman, 1993a; Brimecombe et al., 1997). Voltage dependence of the channel was also unchanged when receptors were treated with reducing and oxidizing agents at physiological potentials (Tang and Aizenman, 1993b). Redox modulation appears to be functionally independent of modulation of the receptor by sulfated steroids (Park-Chung et al., 1997), ethanol (Peoples et al., 1997), and pH (Traynelis and Cull-Candy, 1991; Tang and Aizenman, 1993a). However, dithiothreitol treatment reduced the potency of dynorphin approximately 3-fold for the NMDA receptor (Chen et al., 1995b), and alkylation of the NMDA receptor presumably at thiols that participate in redox modulation has also been suggested to alter Mg^{2+} and Zn^{2+} inhibition (Tang and Aizenman, 1993a). It is noteworthy that the two NR1 cysteine residues that control redox modulation also control inhibition by Zn^{2+}, protons, and ifenprodil (Sullivan et al., 1994; Traynelis et al., 1998; Zheng et al., 1998; Mott et al., 1998).

A host of endogenous molecules have been described that are capable of oxidizing and reducing the NMDA receptor in a functionally relevant manner. These molecules include the oxidizing agents pyrroloquinoline quinone, lipoic acid, and reactive free radical oxygen species (Aizenman et al., 1990; Aizenman et al., 1992; Tang and Aizenman, 1993c; Aizenman, 1995; Scanlon et al., 1997) and reducing agents such as glutathione and dihydrolipoic acid (Gilbert et al., 1991; Manzoni et al., 1992; Tang and Aizenman, 1993b; Köhr et al., 1994; Varga et al., 1997). Nitric oxide (NO) donors also can inhibit the NMDA receptors, perhaps through the release of NO-derived compounds that support *S*-nitrosylation of the NMDA receptor (Lipton et al., 1993; Stamler et al., 1997). However, the exact mechanism of action of NO on NMDA receptors remains controversial (Hoyt et al., 1992; Fagni et al., 1995; Aizenman et al., 1998). Recently, additional compounds such as cyanide have been suggested to exert subunit-specific effects that appear to be linked to chemical modification through the redox site(s) of receptors containing NR2A (potentiation) or NR2B (depression; Arden et al., 1998). The triaminopyridine derivative, flupirtine, may also influence NMDA receptor activation via its redox site (Osborne et al., 1998).

Although a residual NMDA receptor response exists in the oxidized state, the difference between enhanced and oxidized responses is sufficient to suggest involvement in normal function as well as pathological situations (Levy et al., 1990; Puka-Sundvall et al., 1995; Sinor et al., 1997). This degree of regulation of the NMDA receptor by the extracellular redox state has been considered as a potential site for therapeutic intervention in ischemic cell death (Lipton, 1993; Lipton et al., 1993). Furthermore, some compounds that oxidize the NMDA receptor and thereby reduce NMDA receptor activity are anticonvulsant and neuroprotectant in experimental models (Jensen et al., 1994; Quesada et al., 1996, 1997). One important advantage of such modulation is the prospect of diminished side effects since oxidation does not fully inhibit the receptor. However, the feasibility of designing NMDA-specific redox modulators remains to be evaluated.

C. Extracellular Protons

The extracellular pH is highly dynamic in mammalian brain and influences the function of a multitude of biochemical processes and proteins, including glutamate receptor function. AMPA receptors are inhibited by protons at acidic pH values (near 6.0) that make this effect of more biochemical than physiological interest (Christensen and Hida, 1990; Traynelis and Cull-Candy, 1990, 1991; Traynelis et al., 1995). Somatic, postsynaptic (Gottfried and Chesler, 1994; Saybasili, 1998), and presynaptic (Chen et al., 1998) native NMDA receptors are inhibited by more physiologically relevant concentrations of extracellular protons. This inhibition occurs primarily through a voltage- and agonist-independent reduction in the single-channel opening frequency rather than through changes in the single-channel open time or single-channel conductance (reviewed by McBain and Mayer, 1994; Traynelis, 1998). The pH sensitivity of the NMDA receptor has received increasing attention for at least two reasons. First, the IC_{50} value for proton inhibition of exon 5- and NR2C-lacking receptors corresponds to pH 7.4, placing the receptor under tonic inhibition at physiological pH. Second, pH changes are extensively documented in the CNS during synaptic transmission, glutamate receptor activation, glutamate receptor uptake, and also during ischemia and seizures (Siesjo, 1985; Chesler, 1990; Chesler and Kaila, 1992; Amato et al., 1994). The acidification associated with these latter pathological situations should serve to inhibit NMDA receptors, which may provide negative feedback that minimizes their contribution to neurotoxicity (reviewed by Kaku et al., 1993; Munir et al., 1995; Vornov et al., 1996; Gray et al., 1997; O'Donnell and Bickler, 1994; Tombaugh and Sapolsky, 1993) and seizure maintenance (Balestrino and Somjen, 1988; Velisek et al., 1994). Such feedback inhibition might also delay the contribution of NMDA receptor activation to ischemic cell death to a point in time at which the pH

gradient has recovered before glutamate has been removed from the interstitial space. The pH sensitivity of glutamate uptake is consistent with this latter possibility (Billups and Attwell, 1996), which may enhance the opportunity for postinsult treatment of, for example, stroke with NMDA receptor antagonists (Tombaugh and Sapolsky, 1993).

Like voltage-independent Zn^{2+} inhibition, the inhibition of NMDA receptors by protons is also controlled by the NR2 subunit as well as alternative exon splicing in the NR1 subunit (see Traynelis, 1998). Inclusion of NR1 exon 5 reduces both proton and Zn^{2+} inhibition, and the same residues encoded by this exon appear to mediate both effects (see also, Zheng et al., 1994; Traynelis et al., 1995, 1998). These structural parallels extend to other portions of the molecule in that mutations that influence proton inhibition throughout the NR1 subunit similarly influence Zn^{2+} inhibition (Williams et al., 1995; Traynelis et al., 1998; discussed above). Interestingly, mutations that influence pH sensitivity are broad ranging in both NR1 and NR2 and include N-terminal acidic residues (Williams et al., 1995; Gallagher et al., 1997), cysteine residues that may participate in disulfide bond formation (Sullivan et al., 1994), residues in the extracellular M3-M4 loop (Kashiwagi et al., 1996), as well as residues that are thought to comprise the channel pore-forming region (Kashiwagi et al., 1997; Traynelis et al., 1998). This latter association between pore-forming residues and pH sensitivity might suggest that the proton sensor is tightly coupled to the movement of the gate.

What might the molecular composition of the proton sensor be? Whereas it could be lipid (discussed in Traynelis, 1998) or carbohydrate, it seems more likely to be a single amino acid residue that faces the extracellular solution. Cysteine and histidine residues (see Wu and Christensen, 1996) are obvious candidates given the similarity between their free solution side chain pKa values and the IC_{50} for proton inhibition at the NMDA. However, apart from the residues thought to be involved in disulfide bond formation, there are no other cysteine residue substitutions that alter the IC_{50} for proton inhibition (Sullivan et al., 1994). Furthermore, the pKa of a single residue within a protein can differ from its free solution value (see Traynelis, 1998 for examples), raising the possibility that residues with normally acidic or basic pKa values might comprise the proton sensor. For example, the pKa values of clustered intrapore acidic residues, which normally have free solution pKa near 4, are shifted above 7.5 for cyclic nucleotide-gated channels and Ca^{2+} channels (Root and MacKinnon, 1994; Chen et al., 1996; Chen and Tsien, 1997).

Identification of the residues or other molecular entities that constitute the proton sensor seems an important next step, since this information might provide structural clues to NMDA receptor function and regulation. Furthermore, such information might help frame structural models describing how exon 5 of the NR1 subunit (as well as polyamines and Mg^{2+}; see below) acts as a tethered modulator to relieve tonic proton inhibition at the surface of the receptor through shielding of the proton sensor (Paoletti et al., 1995; Traynelis et al., 1995; Johnson, 1996). This information would also help to illuminate the mechanism of ifenprodil's potentiation of proton inhibition (Mott et al., 1998) and could also be useful in the design of novel NMDA receptor antagonists.

D. Extracellular Polyamines

The interactions of endogenous polyamines and polyamine toxins with ion channels has received considerable attention in recent years, both because of the implications for neurophysiology and because of their potential as therapeutic agents. Endogenous polyamines such as spermidine and spermine have at least three effects on NMDA receptors. Extracellular polyamines can cause a voltage-dependent inhibition, a glycine-dependent potentiation, and a voltage- and glycine-independent potentiation of neuronal and recombinant NMDA receptor function (reviewed by Rock and Macdonald, 1995; Williams, 1995a, 1997a,b). The voltage-dependent block appears to involve the same intrapore residues as Mg^{2+} and Zn^{2+} block (Kashiwagi et al., 1997) and likely reflects fast-open channel block that is of lower affinity than Mg^{2+} with relatively weak voltage dependence (e.g., Rock and Macdonald, 1992; Araneda et al., 1992; Benveniste and Mayer, 1993). The voltage-dependent block has a similar subunit dependence as Mg^{2+} blockade, being less pronounced for NR2C-containing receptors when compared with receptors comprised of NR2A or NR2B subunits (Williams et al., 1994; Williams, 1995b). The structure and multivalent nature of the polyamines complicates interpretation of blocking data in terms of a binding site at a particular location within the electric field, although it has been suggested that more than a single charge enters the electric field if the channel behaves as a single ion pore; polyamines may also permeate the channel (Benveniste and Mayer, 1993; Igarashi and Williams, 1995).

Polyamines can stimulate NMDA receptor function to a greater degree at low glycine concentrations than at saturating glycine concentrations. This stimulation reflects approximately a 3-fold increase in glycine affinity (Benveniste and Mayer, 1993). Both glycine-independent and glycine-dependent forms of potentiation of NMDA receptor function are influenced by the NR2 subunit. However, whereas glycine-dependent potentiation occurs at NR2A- and NR2B-containing receptors, glycine-independent potentiation is observed exclusively at receptors that incorporate the NR2B subunit (Zhang et al., 1994; Williams et al., 1994; Williams, 1995b). Consistent with the subunit selectivity, NR1 subunit mutations that influence one process do not perturb the other (Williams et al., 1995; Kashiwagi et al., 1996). Glycine-dependent potentiation is not controlled by NR1 RNA

splicing, whereas glycine-independent potentiation is abolished when the N-terminal alternative exon 5 is incorporated into the mature transcript (Durand et al., 1993). Together, these results suggest that two separate binding sites might exist for glycine-dependent and -independent effects of spermine.

The glycine-independent form of potentiation has been suggested to arise from the relief of tonic proton inhibition at physiological pH. That is, polyamines (like alternative exon 5) shift the pKa of the proton sensor to acidic values, reducing the degree of tonic inhibition at physiological pH, which appears as a potentiation of function (Traynelis et al., 1995; Kumamoto, 1996). Whereas linkage of these two allosteric modulators (protons and spermine) explains their common dependence on splice variants, clearly other structural determinants must be invoked to explain the inability of polyamines to relieve proton inhibition of NR2A containing receptors. These structural determinants of NR2 polyamine potentiation appear to reside within the N terminus of this subunit, and can be attributed in part to several acidic residues that might be involved in polyamine binding (Gallagher et al., 1997).

The role of acidic residues within the NR1 subunit in polyamine-proton regulation has also become apparent through studies utilizing site-directed mutagenesis. Residues first predicted to be involved in polyamine potentiation through comparison of the NR1 sequence to the polyamine-binding protein PotD have also been shown to control proton sensitivity (Williams et al., 1995; Kashiwagi et al., 1996). Thus, mutagenesis through the NR1 and NR2 subunits supports the idea that glycine-independent polyamine potentiation reflects relief of tonic proton inhibition (Traynelis et al., 1995; Williams et al., 1995; Kashiwagi et al., 1996, 1997; Gallagher et al., 1997; Traynelis et al., 1998). Finally, although the physiological relevance of voltage- and glycine-independent polyamine potentiation of NMDA receptor function has remained unclear given the unknown concentrations of extracellular polyamines in vivo, recent data have identified two endogenous activators of the polyamine site, Mg^{2+} and histamine. Mg^{2+} acts with an IC_{50} value of 2 mM to partially reduce the pH sensitivity of NR2B-containing receptors under physiological conditions (Paoletti et al., 1995; Kumamoto, 1996). Histamine can act with an EC_{50} near 10 μM to potentiate neuronal and synaptic NMDA receptors (Vorobjev et al., 1993; Bekkers, 1993). This effect was originally suggested to involve polyamine potentiation on the basis of the nonadditive effects of spermine (Vorobjev et al., 1993). In addition, both the subunit and pH dependence of histamine potentiation support the idea that histamine potentiates NMDA receptors through action at the polyamine site (Williams, 1994; Yanovsky et al., 1995; Saybasili et al., 1995). Because histamine is released from widespread synaptic varicosities arising from the anterior hypothalmus, this form of regulation may be relevant under physiological conditions. Finally, aminoglycoside antibiotics may also mimic the potentiating effects of polyamines, which might contribute to the ototoxicity observed with these compounds (Segal and Skolnick, 1998).

In summary, the last few years have seen the evaluation and considerable refinement of ideas about allosteric regulation in recombinant NMDA receptors. In addition to the continued discovery of new forms of regulation, one interesting trend to emerge from work on voltage-independent modulation of NMDA receptor function has been the convergence of regulatory systems. For example, site-directed mutagenesis has been used to suggest structural links between proton, zinc, polyamine, and redox modulation of the NMDA receptor. Although it would be oversimplistic to argue that these sites are identical, there is strong evidence to suggest that they may share either partially overlapping binding determinants or common downstream structural targets. Although convergence remains to be evaluated among the other modulatory systems, there is evidence that not all regulatory sites share structural and functional principles (Miller et al., 1992; Nishikawa et al., 1994; Chen et al., 1995b; Park-Chung et al., 1997; Peoples et al., 1997). The next few years are likely to be pivotal in identifying subgroups within Table 3 that share common features. This advance should facilitate the design of drugs that can modulate rather than fully abolish NMDA receptor function.

VIII. Molecular Determinants of Ion Permeation

From the wealth of biophysical data and recent structural information, the mechanisms and molecular determinants of ion permeation are best understood for voltage-gated Ca^{2+}, Na^+, and K^+ channels that discriminate among ions with a high degree of selectively. Traditionally, biophysical experiments suggest that selectivity is not governed simply by the physical constraints of the pore in a manner analogous to a sieving effect, but rather, ions may occupy several binding sites in the pore which are electrostatically coupled. Such an arrangement permits rapid ion transport through the channel while maintaining a high degree of selectivity. The recent elucidation of K^+ channel structure from *Streptomyces lividans* (Doyle et al., 1998) has provided structural information that creates a framework in which to interpret biophysical information in the future. In contrast, the mechanism of permeation and block of less selective channels, such as glutamate-activated receptors, remains unclear. Yet, understanding such mechanisms is important since permeation and block are controlled in a cell-specific manner through differential subunit expression and RNA editing. In view of this, we consider here the available biophysical data and recent mutagenesis studies of glutamate receptor pores that provide information on the pore diameter, single-chan-

nel conductance properties, and ion selectivity as well as block by endogenous ions such as Mg^{2+} or polyamines.

A. Pore Diameter

Traditionally, the minimum pore diameter of ion channels has been inferred from studies of permeating metal ions of differing hydrated radii or using organic ions with known space-filled volumes (Hille, 1992). Table 5 summarizes estimates of the minimum pore diameter for recombinant glutamate receptors determined by comparing the permeability sequence of a series of organic cations. Unlike most voltage-gated channels (see Table 5), the pores of glutamate receptors are physically larger, approximating the dimensions of nonselective endplate channels that may function in part as molecular sieves (Hille, 1992). Moreover, the dimensions estimated for the open channel of NMDA and non-NMDA receptors are large enough to account for permeation of Mg^{2+} ions at negative membrane potentials or polyamines at positive potentials (Bähring et al., 1997; Bowie et al., 1999).

Additional studies with impermeant organic cations also suggest that both NMDA and non-NMDA receptors have a short narrow region of constriction somewhere near or just past the middle of the membrane electric field, which is flanked by two wider vestibules that can accommodate cations as large as 0.73 nm (Fig. 6; Zarei and Dani, 1995; Villarroel et al., 1995). This hypothesized geometry is in agreement with the accessibility profile of cysteine-substituted residues (Kuner et al., 1996, 1997; discussed below) and differs somewhat from that of the axial fluctuations in pore diameter of K^+ channels (Doyle et al., 1998), even though glutamate and K^+ channels have been suggested to share some other features (Lee, 1992; Wood et al., 1995).

This geometry raises the possibility that NMDA channels may behave as single-occupancy pores with respect to the main permeant ion (Schneggenburger, 1996). However, exceptions to this idea seem to exist for certain organic blockers since the NMDA receptor apparently can be occupied by both blocking and permeant ions simultaneously (Antonov et al., 1998). Consistent with the properties of single-ion pores, the unitary conductance reaches a sustained maximum in the absence of divalent ions of 90 pS with increasing concentrations of NH_4^+ as the charge carrier for hippocampal NMDA receptor (Zarei and Dani, 1994; Jahr and Stevens, 1993) and 60 to 160 pS with Na^+ as the charge carrier for recombinant NMDA receptors (Ruppersberg et al., 1994; Premkumar and Auerbach, 1996; Iino et al., 1997). This is in contrast to the multiphasic concentration-conductance curve that shows a minimum predicted for dual occupancy pores (see Hille, 1992). Whereas experiments with monovalent ions suggest that the biophysical basis of ion transport may differ between NMDA receptors (single-occupancy pores) and voltage-dependent Ca^{2+} channels (dual occupancy pores; discussed in Zarei and Dani, 1994; see Table 5), Ca^{2+} permeation appears to be more complex and may involve multiple binding sites (Premkumar and Auerbach, 1996; Wollmuth and Sakmann, 1998). Interestingly, mutation of residues within the glutamate receptor channel that control unitary conductance, Ca^{2+} permeability, and sensitivity to blockade by polyamines has little effect on the pore's minimum cross-sectional area (Table 5), suggesting that the electrostatics or coordination chemistry of permeant ions rather than purely steric considerations within the pore govern the functional effects of RNA editing on unitary conductance and ion selectivity.

B. Unitary Sublevel Conductances

One of the most intriguing features of the glutamate receptors apparent from the first single-channel studies in native channels (Nowak et al., 1984; Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987) is the striking variability in sublevel conductances of the open

TABLE 5

Comparison of pore dimensions of glutamate receptors determined by permeability to organic cations to measurements from other cation selective channels

Channel or Receptor	Pore Dimensions	Pore Area	References
	nm	*nm²*	
Neuronal AMPA[a]	0.6 diameter	0.28	Vyklicky et al., 1988
Neuronal NMDA[b]	0.45 × 0.57	0.26	Zarei and Dani, 1995
GluR1[c]	0.78 diameter	0.48	Burnashev et al., 1996
GluR1/GluR2	0.70 diameter	0.38	Burnashev et al., 1996
GluR6(Q)[c]	0.75 diameter	0.44	Burnashev et al., 1996
GluR6(R)[c]	0.76 diameter	0.45	Burnashev et al., 1996
GluR6(Q)/GluR6(R)	0.74 diameter	0.43	Burnashev et al., 1996
NR1-1a/NR2A	0.55 diameter	0.24	Villarroel et al., 1995; Wollmuth et al., 1996
$5HT_3$, serotonin[d]	0.76 diameter	0.45	Yang, 1990; Yakel et al., 1990
Muscle nicotinic	0.65 × 0.65	0.42	Dwyer et al., 1980
Na^+ Channel in frog nerve	0.31 × 0.51	0.16	Hille, 1992
K^+ Channel in frog nerve	0.17 diameter[e]	0.09	Hille, 1973; see Doyle et al., 1998
Ca^{2+} Channel in frog muscle	0.6 diameter[e]	0.28	McCleskey and Almers, 1985

[a] Cultured chick cortical and diencephalic neurons.
[b] Cultured rat hippocampal neurons.
[c] Homomeric receptors.
[d] N18 neuroblastoma cells.
[e] Diameter calculated from area.

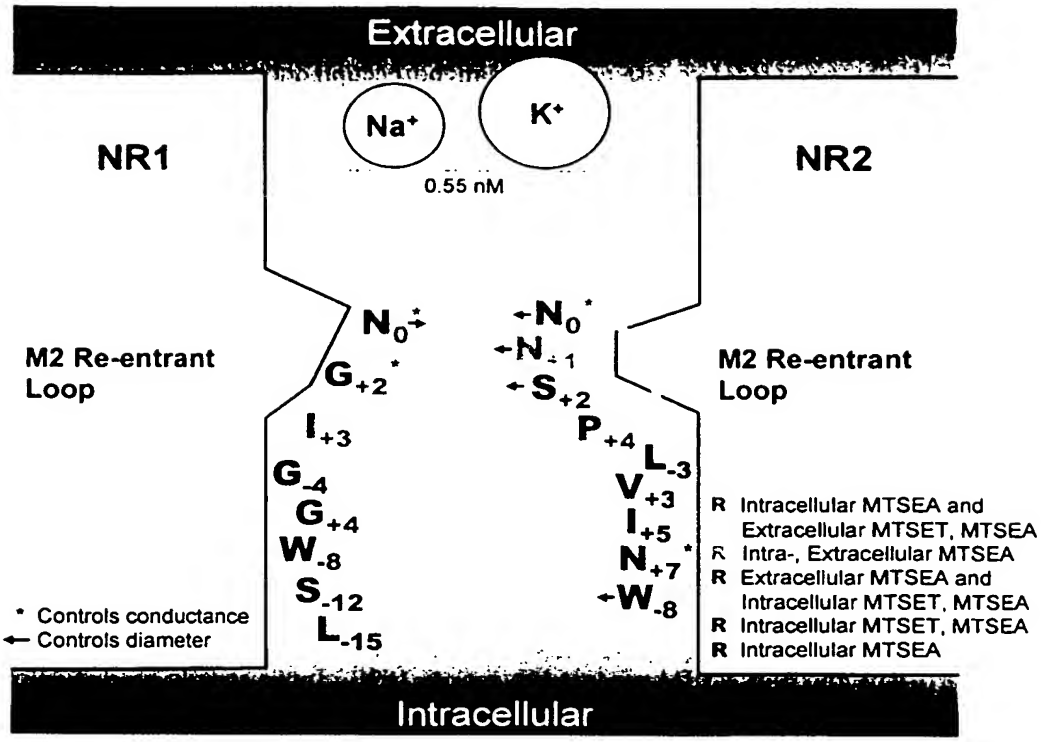

FIG. 6. Cysteine-substituted residues in the NMDA receptor pore that are accessible to sulfhydryl reagents. The pore-forming region of the NMDA receptor is shown, with the extracellular solution and vestibule shaded red and the intracellular solution and vestibule shaded blue. Residues that are reactive with extra- or intracellular application of the thiol-modifying drugs methanethiosulfonate-ethyltrimethylammonium (MTSET; charged head group diameter 0.58 nm) and methanethiosulfonate-ethylammonium (MTSEA; charged head group diameter 0.36 nm) are shown as indicated by the color code. Residues that have been shown or suggested to control the narrow constriction of the pore are indicated by arrows. Residues at which mutations are known to alter single-channel conductances are marked with an asterisk. Figure summarizes unitary conductance data from Behe et al. (1995), Kupper et al. (1996), Premkumar and Auerbach (1997), Sharma and Stevens (1996a). Pore diameter data are from Kashiwagi et al. (1997) and Wollmuth et al. (1996, 1998a). Thiol-reactive cysteine mutageneisis data are from Kuner et al. (1996). The numbering scheme for M2 residues is that of Kuner et al. (1996), where N_0 in NR1 is Asn616 and N_0 in NR2 corresponds to Asn614 for NR2A using the initiating methionine as residue 1 rather than the cleavage site for the signal peptide, which has been assigned variable positions by different groups. Na^+ and K^+ ions shown using Pauling radii. The narrow constriction (0.55 nm; Table 1) is placed about 50% through the electric field (Zarei and Dani, 1995).

state both within a given cell type and across different preparations. Do the widely varying conductance levels observed result solely from multiple receptors or do they also reflect in part the properties of individual glutamate receptors? It now appears that most sublevels first observed in neurons can be accounted for in recombinant systems. The extreme variation in channel conductance (e.g., 0.2 versus 50 pS) for native non-NMDA receptors seems largely attributable to variable RNA editing of GluR5 or GluR6 (kainate receptors) and variable expression of fully edited GluR2 (AMPA receptors). The presence of arginine in the Q/R site lowers single-channel conductance substantially (Howe, 1996; Swanson et al., 1996, 1997; Traynelis and Wahl, 1997; Pemberton et al., 1998). A smaller contribution to variation in conductance levels is produced by subunit selection (Table 6). In addition, the varied conductance levels (and transitions between levels) of NMDA receptors observed in native neurons (Cull-Candy and Usowicz, 1987; Cull-Candy et al., 1988; Momiyama et al., 1996) closely match the multiple conductance levels of heteromeric combinations of NR1 and various NR2 subunits (Table 6). The multiple conductance levels of native non-NMDA receptors (Wyllie et al., 1993) also show strong similarities with recombinant receptors (Table 6). Thus, the multisublevel conductances of glutamate receptors can be recapitulated in recombinant systems, providing perhaps the best evidence that sublevels arise from within the same receptor complex rather than from a heterogeneous mix of different channels expressed by neurons. Moreover, distinct receptors differing by only a single amino acid as a result of RNA editing appear likely to account for the wide variation in conductances observed across neuronal preparations.

Despite careful description of the conductance levels of neuronal and recombinant NMDA receptors, only marginal headway has been made toward understanding the basis for the different subconductance levels. A variety of possibilities have been raised as explanations for subconductance levels in general, including long-lived conformations with distinct pore properties, channels with multiple pores, channels exhibiting periods of rapid transition between open and closed states, alterations in electrostatic properties of the pore, protonation of residues that control ion transport, and physical changes in pore dimensions (Lauger, 1985; Fox, 1987;

TABLE 6
Single-channel conductance levels for various glutamate receptor subunit combinations

Receptor	pS	%	pS	%	pS	%	References
GluR2flip[a,b]					0.4		Swanson et al., 1997b
GluR4flip[b]	24	8	15	18	8	74	Swanson et al., 1997b
GluR4flip/GluR2			10	8	4	92	Swanson et al., 1997b
GluR5(R)[b–d]					0.2		Swanson et al., 1996
GluR5(R)/KA2[d]					1.0		Swanson et al., 1996
GluR5(Q)[b, d]	14	12	9	67	5	21	Swanson et al., 1996
GluR5(Q)/KA2[d]	17	5	9	18	5	77	Swanson et al., 1996
GluR6(R)[b, d, e]					0.35		Howe, 1996; Swanson et al., 1996; Traynelis and Wahl, 1997
GluR6(R)/KA2[d, e]					0.65		Howe, 1996; Swanson et al., 1996
GluR6(Q)[b, d]	25	4	15	15	8	81	Swanson et al., 1996
GluR6(Q)/KA2[d]	20	6	12	20	7	74	Swanson et al., 1996
NR1-1a/NR2A	51	77	38	23			Stern et al., 1992, 1994
NR1-1a/NR2B	51	83	39	17			Stern et al., 1992
NR1-1a/NR2C			36	75	19	25	Stern et al., 1992
NR1-1a/NR2D			35	61	17	39	Wyllie et al., 1996
NR1-1a/NR2A/N	75	ND	35	ND			Das et al., 1998

The three largest slope or chord single-channel conductance levels are shown for steady-state activation of recombinant glutamate receptors. Unless stated otherwise, glutamate (GluR1-6) or glutamate plus glycine (NR1/NR2A-D) were applied and responses were recorded in 0.85 to 1.0 mM external Ca^{2+}. The relative proportion of openings to each level is indicated as percent. ND, not determined.

[a] Similar results were obtained for both GluR2flip and GluR2flop.
[b] Homomeric receptors.
[c] Receptors were pretreated with concanavalin A.
[d] Receptors were activated by domoate.
[e] Receptors were activated by kainate; conductance was determined by variance analysis.
[f] Channel openings were recorded nM levels of extracellular Ca^{2+}; conductance levels may arise from separate channels.

Dani and Fox, 1991; Root and MacKinnon, 1994; Schneggenburger and Ascher, 1997). Although it remains unclear whether subconductance levels arise from physical changes in the pore or other more subtle changes in protein conformation, at least NMDA receptor subconductance levels do not appear to reflect rapid transitions between the open or closed state, multiple conductance paths (Premkumar et al., 1997), or protonation (Traynelis and Cull-Candy, 1991).

How might the pore properties be modified to account for subconductance levels? One interesting approach to this question relies on experiments in which all subunits of a homomeric receptor are bound by a competitive antagonist, and the dissociation of antagonist from one site (i.e., one subunit) at a time is studied in the presence of saturating agonist to evaluate the contribution of multiple agonist-binding steps to receptor function. Rosenmund et al. (1998; discussed above) have used this method to suggest that the different conductance states of homomeric GluR3 receptors might arise from channels with different numbers of bound ligands. If this interpretation proves correct (and antagonist binding does not alter subunit function), then these data suggest that consecutive ligand binding might ratchet open the pore to different dimensions or otherwise create a pore of uniform dimension but with incrementally changing properties. This might lead to different ionic permeabilities of the subconductances since the pore itself would be different. One indication that this might be true for glutamate receptors is the correlation between the unitary conductances and Ca^{2+} permeabilities among NMDA receptors containing different NR2 subunits (Burnashev et al., 1995; Stern et al., 1992; compare Tables 6 and 7). Another indication that subconductance levels possess different ionic selectivities comes from work with mutant NMDA receptors containing NR1(N616Q), which exhibit two sublevels with different monovalent ionic selectivities (Schneggenburger and Ascher, 1997). Similar results also have been reported for mutant *Shaker* potassium channel sublevels (Zheng and Sigworth, 1997). Study of this same NR1 mutant subunit coexpressed with NR2A containing a similar mutation showed different sensitivity to divalent block between the two subconductance levels (Premkumar and Auerbach, 1996; Premkumar et al., 1997). Interestingly, similar to studies with channel-blocking compounds that suggested occupancy of the permeation path can alter channel gating (Antonov and Johnson, 1996; Bowie et al., 1998), Schneggenberger and Ascher (1997) show that permeant ions can also influence gating under certain conditions.

Do receptors activated by the rapid synaptic release of glutamate open to the same conductance levels as those produced by steady-state application of other receptor agonists (e.g., domoate, AMPA, kainate, quisqualate)? One useful approach to this question is nonstationary variance analysis (Traynelis and Jaramillo, 1998), which can be used to evaluate the weighted mean conductance of the channels that constitute the response to synaptically released glutamate. Using this method, a variety of investigators have measured a weighted mean conductance of 6 to 29 pS for native non-NMDA channels that open in response to high concentrations of rapidly applied glutamate, conditions that mimic synaptic transmission (Hestrin, 1992; Jonas et al., 1994; Raman and Trussell, 1995; Spruston et al., 1995; Koh et al., 1995b). Nonstationary variance analysis of synaptic currents mediated by non-MDA receptors has provided sim-

ilar conductance estimates (Traynelis et al., 1993; Silver et al., 1996), and also the surprising result that synaptic conductance might increase in some forms of plasticity (Benke et al., 1998). Although these conductance values are not directly comparable to those obtained from excised patches exposed to non-natural agonists, they are at least similar in range (see Table 6).

C. Ionic Selectivity

Glutamate receptors are permeable to cations and, with the exception of homomeric GluR6 and GluR2 (Table 7), largely exclude anions from the pore. Sodium and potassium are thought to be nearly equally permeable, and thus extensive comparisons between the two ions have not been made. However, because Ca^{2+} has the ability to couple electrical to biochemical signaling and alter intracellular ion concentrations (Brocard et al., 1993), its permeability through various glutamate receptors has received considerable attention. Consistent with previous results in neuronal glutamate receptors (McBain and Mayer, 1994), recombinant NMDA receptors appear to be more permeable to Ca^{2+} than non-NMDA glutamate receptor subtypes (Table 7) and other cation-selective receptors (Rogers and Dani, 1995). Ca^{2+} permeation is more complex in NMDA than AMPA receptors, and the increased Ca^{2+} permeability of NMDA receptors may reflect the presence of multiple intrapore Ca^{2+}-binding sites, which could cooperate to enhance Ca^{2+} flux (Premkumar and Auerbach, 1996; Wollmuth and Sakmann, 1998). NMDA receptors also possess lower unitary conductances in high Ca^{2+} (Jahr and Stevens, 1987; Ascher and Nowak, 1988; Gibb and Colquhoun, 1992; Iino et al., 1997; Wyllie et al., 1996), as expected if Ca^{2+} hesitates longer in the channel and thus permeates more poorly than monovalent ions. Interestingly, AMPA receptors lacking the GluR2 subunit (for which the Q/R site codon is almost 100% edited to one encoding arginine; see above) are also Ca^{2+} permeable (see Table 7), as are kainate receptors with glutamine in the Q/R site of the M2 region. Since the elucidation of the molecular determinants of Ca^{2+} permeability in AMPA receptors (Hollmann et al., 1991; Hume et al., 1991; Burnashev et al., 1992a), there have been a wide range of reports demonstrating Ca^{2+}-permeable AMPA receptors in identified neurons and glia (Muller et al., 1992; McBain and Dingledine, 1993; Jonas et al., 1994; Geiger et al., 1995; Otis et al., 1995; Koh et al., 1995b; Zhang et al., 1995; Steinhauser and Gallo, 1996 and references therein; Washburn et al., 1997). The extensive regulation of Ca^{2+} permeability by RNA editing and cell-specific GluR2 expression underscores the importance of this property for neuronal and glial function. Moreover, the unedited receptors expressed by interneurons are typically rapidly gated compared with receptors in principal neurons (Geiger et al., 1995), which may contribute to the burst-firing patterns of many interneuron populations.

Mutagenesis of residues that impact permeation properties has led to partial understanding of the structural basis for ionic selectivity. The genomic codon for Gln607 in the GluR2 primary transcript is almost completely edited in the mRNA to an arginine codon (see above), and inclusion of even a single copy of this subunit in the receptor complex appears to render the resulting receptors less Ca^{2+} permeable (Geiger et al., 1995; Washburn et al., 1997). Likewise, editing of the codon at Gln621 of GluR6 reduces Ca^{2+} permeability (Table 7); editing in both receptor classes also reduces the single-channel conductance (Table 6). Interestingly, the presence of the positively charged arginine within the GluR6 pore causes a substantial increase in Cl^- permeability (Table 7). After these studies of non-NMDA receptors, homologous residues in the NR1 (Asn616) subunit and the homologous and adjacent asparagine residues in the NR2 subunit (Asn614 and 615 in NR2A) of the NMDA receptor were found to influence Ca^{2+} permeability (Burnashev et al., 1992b; Kawajiri and Dingledine, 1993; Sakurada et al., 1993; Wollmuth et al., 1996; Schneggenburger, 1998) as well as single-channel conductance (Behe et al., 1995; Premkumar and Auerbach,

TABLE 7

Ionic permeabilities relative to monovalent ions for glutamate receptors of known subunit composition

Receptor	$P_{Cs}/P_{Na,Cs,K}$[a]	P_{Cl}/P_{Na}[a]	$P_{f(Ca)}$[b]	
GluR1	2.34		0.032	Burnashev et al., 1995
GluR2		0.14		Burnashev et al., 1996
GluR1/GluR2	0.05	~0	0.005	Burnashev et al., 1995, 1996
GluR4			0.039	Burnashev et al., 1995
GluR6(R)[f]	0.12[d]	0.74	0.002[c]	Burnashev et al., 1995, 1996
GluR6(Q)	0.21–1.2	~0	0.016[c]	Egebjerg and Heinemann, 1993; Burnashev et al., 1995, 1996
GluR6(R)/GluR6(Q)		~0	0.006[f]	Burnashev et al., 1995, 1996
NR1-1a/NR2A	3.10–11	0.1–0.19		Burnashev et al., 1995; Wollmuth et al., 1996; Sharma and Stevens, 1996b; Schneggenburger, 1996, 1998
NR1-1a/NR2B	17		0.175	Iino et al., 1997; Schneggenburger, 1996
NR1-1a/NR2C	2.23		0.082	Burnashev et al., 1995

Na^+ and Cs^+ are considered equally permeable.
[a] Lewis equation derived from the Goldman-Hodgkin-Katz equation.
[b] Proportion of whole-cell current carried by Ca^{2+} determined from simultaneous measurement of whole-cell current and Ca^{2+} fluorescence.
[c] Results are shown only for studies which either directly measure Ca^{2+} flux or take into consideration the Cl^- permeability.
[d] Not significantly different from zero.
[e] Editing of transmembrane region 1 had no effect fractional Ca^{2+} current through GluR6(R), but slightly decreases fractional Ca^{2+} current through GluR6(Q).
[f] Expressed at a ratio of 1:1.

1996). In addition, mutations at acidic residues near the C-terminal end of M2 (see also discussion of GluR3 Asp616 in Dingledine et al., 1992; Chazot et al., 1993; NR1 Glu621, Schneggenburger, 1998) as well as a leucine and tryptophan residue (e.g., GluR1 Leu592, NR1 Trp611, NR2B Trp 607; Ferrer-Montiel et al., 1996, 1998; Williams et al., 1998) in or near M2 in NR1 also control Ca^{2+} permeability. In AMPA receptors, replacement of arginine by the smaller (but still positively charged) lysine in the Q/R site apparently increased Ba^{2+} permeability, as expected if this residue influences permeation (Dingledine et al., 1992).

Thus, in all three classes of glutamate receptor there are strong data arguing that structural perturbations at the Q/R/N site and several nearby residues within the M2 region of the receptor alter ion selectivity. M1 may also contribute to selectivity, although effects are not striking. The simplest interpretation of these data is that these critical residues all reside in the pore of the receptor. In such a case, either the side chains or main chains may interact with passing ions or help define the environment and conformation of the pore.

IX. Molecular Determinants of Channel Block

A. External Mg^{2+} Block of NMDA Receptors

One unique feature of the NMDA receptor compared to other ligand-gated ion channels is the dual dependence of function on agonist binding and membrane potential. This property renders the Ca^{2+} flux through NMDA receptors a coincidence detector for depolarization and synaptic release of glutamate. Interestingly, the NMDA receptor's voltage dependence follows directly from channel block by submillimolar concentrations of extracellular Mg^{2+} rather than from the voltage dependence of conformational changes (Nowak et al., 1984; Mayer et al., 1984; Jahr and Stevens, 1990a,b). Binding of extracellular Mg^{2+} within the pore is strongly voltage-dependent, and this property dominates the physiological role of NMDA receptors. At resting membrane potentials, most (but not all; see below) subtypes of NMDA receptor undergo rapid channel block by extracellular Mg^{2+}, which reduces the NMDA receptor component of synaptic currents considerably. However, when neurons are depolarized, for example, by intense activation of colocalized postsynaptic AMPA receptors, the voltage-dependent block by Mg^{2+} is partially relieved, allowing ion influx through activated NMDA receptors. The resulting Ca^{2+} influx can trigger a variety of intracellular signaling cascades, which can ultimately change neuronal function through activation of various kinases and phosphatases.

Based on the measured voltage dependence of block, Mg^{2+} has been proposed to bind to a site deep within the pore, probably near or past the middle of the electric field (Ascher and Nowak, 1988; Johnson and Ascher, 1990; Ruppersberg et al., 1994; Zarei and Dani, 1994; Premkumar and Auerbach, 1996; Wollmuth et al., 1998a). For receptors containing NR2A or NR2B, Mg^{2+} would bind with a voltage-dependent K_d near 10 μM at -80 mV; Mg^{2+} potency at 0 mV is much lower, with a K_d of 2 to 7 mM (Wollmuth et al., 1998a). NR2C receptors are ~10-fold less sensitive (Monyer et al., 1992, 1994; Ishii et al. 1993), and the structural determinants of this decreased sensitivity appear to reside within M1, M4, and the intervening M2-M3 linker (Kuner and Schoepfer, 1996). At resting potentials, block rapidly reaches equilibrium within the duration of an NMDA receptor burst such that individual blockages and unblockages are readily apparent in single-channel records. At very hyperpolarized potentials (or in the absence of other ions; Stout et al., 1996), Mg^{2+} can be driven through the channel, suggesting that it is a permeant blocker (e.g., Mayer and Westbrook, 1987; Ascher and Nowak, 1988; Wollmuth et al., 1998a). The permeability of various divalent cations inversely correlates with their energies of dehydration (Mayer and Westbrook, 1987; Ascher and Nowak, 1988), suggesting that ions with tightly bound water have difficulty passing through the channel. The idea that permeation requires dehydration fits well with the size of hydrated blocking ions such as Mg^{2+} (0.64 nm) and the size of the NMDA receptor pore (<0.6 nm in diameter; see Table 5). Although highly permeable Ca^{2+} and poorly permeable Mg^{2+} appear to bind to separate sites on the basis of their voltage dependence of block, the extent of channel block of wild-type and mutant receptors by Mg^{2+} can be altered by extracellular Ca^{2+} (Mayer and Westbrook, 1987; Sharma and Stevens, 1996b; discussed in McBain and Mayer, 1994). Similarly, the voltage dependence can also be influenced by different intracellular ions (Ruppersberg et al., 1994).

The structural determinants of external Mg^{2+} block are strikingly similar to those governing Ca^{2+} permeability through NMDA receptors; a similar situation is also apparent in non-NMDA receptors with comparison of polyamine blockers and Ca^{2+} permeability (see below). Whereas individual substitutions may have differing effects on Ca^{2+} permeability versus Mg^{2+} block, the same residues typically influence both (Fig. 7). Residues critical for extracellular Mg^{2+} block fall into two categories: a polar Q/R/N site and a nearby hydrophobic site. Substitutions at the Q/R/N site on NR1 or at similar positions on NR2 can relieve Mg^{2+} blockade (Burnashev et al., 1992b; Mori et al., 1992; Sakurada et al., 1993; Kawajiri and Dingledine, 1993; Sharma and Stevens, 1996a; Wollmuth et al., 1998a). For example, substitution of arginine for asparagine at residue 616 of NR1 reduced Mg^{2+} blockade more than substitution of glycine, serine, glutamine, or aspartate. In contrast, the same substitutions at NR2A Asn615 all strongly reduced Mg^{2+} block (Wollmuth et al., 1998a). These results have been interpreted to suggest that the asparagine residue on NR2A may be more critical for block, perhaps by participating in Mg^{2+} chelation. Mg^{2+} block

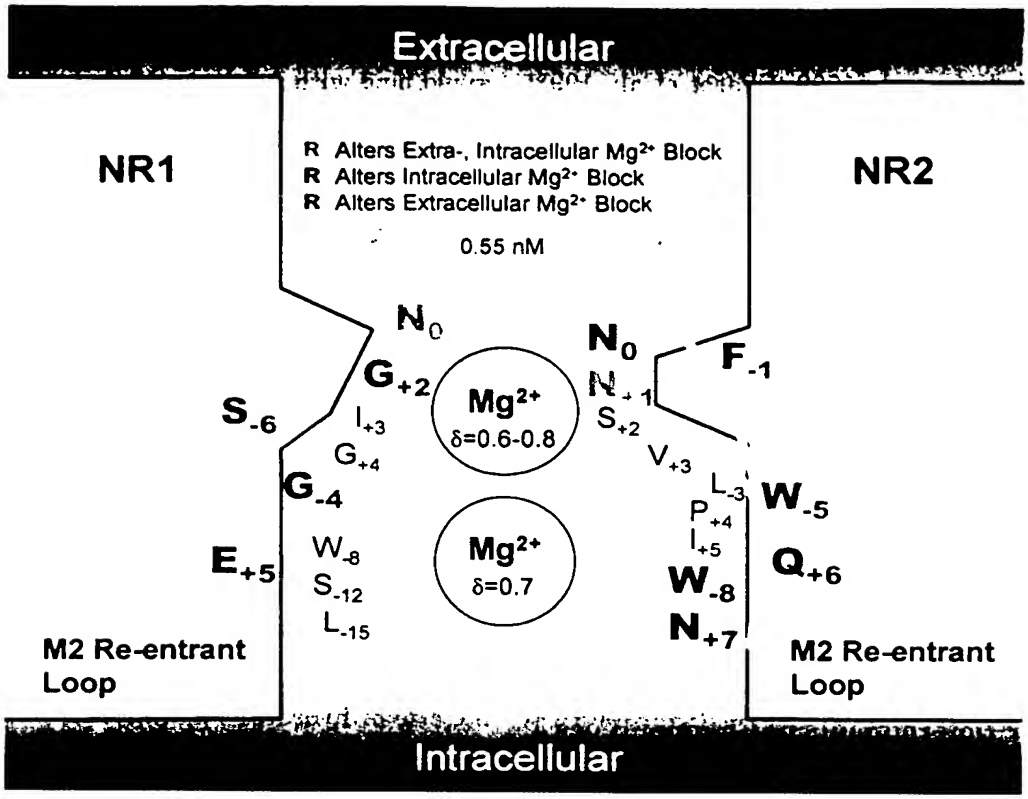

FIG. 7. Residues involved in block of the NMDA receptor by Mg^{2+}. The pore-forming region of the NMDA receptor is shown, with the extracellular solution and vestibule shaded red and the intracellular solution and vestibule shaded blue. All residues are shown for NR2A although experiments supporting their accessibility and control of voltage-dependent Mg^{2+} block were performed in receptors containing NR1 plus NR2A, NR2B, or NR2C subunits. Thus, we ignore NR2 subunit variations in pore conformation and Mg^{2+} blockade, as well as the possibility that different copies of the same subunit in the receptor complex may contribute complementary yet distinct subsets of these residues to the pore (i.e., N_0 and N_{-1} might be on one NR2 subunit and S_{-2} and P_{+4} might be contributed by a second NR2 subunit). Residues that are presumably accessible to the pore are shown within the pore (Kuner et al., 1996; Fig. 6), even though they may contribute their main chain carbonyl groups rather than side chains to the pore environment. Bold indicates residues at which mutations have been shown at physiological potentials to reduce the voltage dependence of Mg^{2+} block, reduce the affinity of Mg^{2+} block, or increase Mg^{2+} permeability. Block by intra- and extracellular Mg^{2+} was not examined in all residues shown. Residues are color-coded to indicate whether they control extracellular Mg^{2+} blockade (red), intracellular Mg^{2+} blockade (blue), or both (purple). This figure summarizes data from Kuner et al. (1996), Kupper et al. (1996, 1998), Williams et al. (1998), and Wollmuth et al. (1998ab). δ is the distance through the electric field from outside to inside and is from Wollmuth et al. (1998a) and Li-Smerin and Johnson (1996); Mg^{2+} shown using Pauling radius.

does not seem to be determined by pore size, although the voltage dependence of block may be influenced by increased permeability of Mg^{2+} in mutant receptors with larger pore diameters (Wollmuth et al., 1998a).

Williams et al. (1998) have recently shown that certain tryptophan residues in the M2 region of NR2 also control permeability and block by Mg^{2+}. Exchange of tryptophan residues on NR2B (Trp607) and NR2A (Trp606) for nonaromatic residues (Ala, Asn, or Leu) reduced Mg^{2+} blockade, whereas similar mutations on NR1 had no effects. When Trp607 in NR2B was mutated to large hydrophobic residues (Y or F), there was no effect on Mg^{2+} IC_{50} at -70. These latter substitutions suggest that a large hydrophobic residue at this position is critical for Mg^{2+} block. These authors speculate that π bonding of delocalized electrons to the metal might be important determinants of Mg^{2+} binding, and that this residue might constitute a barrier predicted from modeling to exist between the intra-and extracellular Mg^{2+}-blocking sites (Li-Smerin and Johnson, 1996; Kashiwagi et al., 1997). However, structural data do not support an interaction between the required consensus sequence for K^+ channel selectivity and permeating ions, suggesting that the molecular nature of the effects of mutations at Trp606/607 in the NR2 subunits requires more investigation.

The chemical nature of the intrapore Mg^{2+}-binding site is impossible to ascertain from any of the standard approaches presently used to study channel block. Nevertheless, numerous authors have proposed that the Q/R/N site and certain tryptophan residues in the NMDA receptor (NR1 Asn616, NR2A Asn 615, NR2B Trp607) physically bind to Mg^{2+} (Mori et al., 1992; Kawajiri and Dingledine, 1993; Sakurada et al., 1993; Sharma and Stevens, 1996a; Williams et al., 1998; Wollmuth et al., 1998a); the available evidence supporting NR2A-asparagine interaction with Mg^{2+} is perhaps the most compelling (Wollmuth et al., 1998a). What this means for asparagines is that a lone pair of electrons from the oxygen within the amide group would satisfy

one of the hybrid orbitals that dictate Mg^{2+} coordination chemistry. Extensive π bonding in aromatic residues such as tryptophan can also coordinate metals (Kumpf and Dougherty, 1993). If there is a ring of similar amide or aromatic groups contributed from NR1 and NR2 subunits at roughly the same position in the pore, they should be able to satisfy some of the coordination sites of Mg^{2+}. Alternatively, main chain carbonyls may particpate in coordination of Mg^{2+} at its binding site.

One might not expect full coordination of the permeating ion by the protein given the correlation between dehydration energies and lack of permeability for various divalent ions (Mayer and Westbrook, 1987; Ascher and Nowak, 1988). The correlation implies that Mg^{2+} is poorly permeable because it does not easily shed its hydration shell and remains as a hydrated complex that is too big to permeate. However, because the dehydration energy is a function of the charge-to-surface area ratio of an ion, it is also a reflection of the small diameter of Mg^{2+}. Thus, it is possible that fully dehydrated Mg^{2+} might be exactly the right diameter to form a coordination complex with geometrically fixed intrapore asparagine and/or tryptophan residues or other electron donors. In this case, the diameter of other divalent ions would dictate their ability to coordinate with this site in the pore. If coordination with such a site (and thus channel block) was dependent on small diameter, permeability would appear to be correlated with low energy of dehydration. Consistent with this notion is the finding that mutations that increase the pore diameter do not always alter Mg^{2+} blockade (Wollmuth et al., 1998a). In any model, it is important to remember that the molecular nature of the pore is uncharted and probably does not fit conventional notions of a water-filled space with a dielectric constant near $80\epsilon_o$. New techniques and structural data will be required to evaluate the nature of the complex interactions of Mg^{2+} with pore-forming residues.

B. Internal Mg^{2+} Block of NMDA Receptors

Intracellular Mg^{2+} can also exert a voltage-dependent block of NMDA receptor channels (Johnson and Ascher, 1990). Assuming that the voltage dependence of block reflects only the position of the blocker in the membrane electric field, the blocking site for internal Mg^{2+} lies ~35% of the electric field from the intracellular side (Johnson and Ascher, 1990; Wollmuth et al., 1998b). The dissociation constant for Mg^{2+} binding is 8 mM at 0 mV, similar to that calculated for extracellular block (Ascher and Nowak, 1988). However, the absence of resolvable blocked states with internal Mg^{2+} (i.e., flickers) reflects a weaker voltage dependence of blocker affinity that is observed experimentally as an apparent reduction in single-channel current amplitudes (Johnson and Ascher, 1990). The proposed electrical distances of the internal and external Mg^{2+} sites present a problem in that access to each site entails either complete or near crossing of the other. Resolution of this latter problem requires new models of channel block (Ruppersberg et al., 1994) or revision of our interpretation of δ as a measure of the relative position of the blocker in the electric field (Woodhull, 1973). One possible explanation to account for this dichotomy is that Mg^{2+} occupancy may be coupled to movement of the permeant ion from its site (i.e., the narrow constriction), which would steepen the apparent voltage dependence of external block (Zarei and Dani, 1994, 1995; Antonov et al., 1998). Other possibilities include the presence of a reduced dielectric constant within a portion of the pore that might increase the effectiveness with which a charge senses an electric field. Ion-ion interactions between permeating and blocking ions within the pore and permeation by Mg^{2+} upon unbinding can also complicate the interpretation of the measured values for δ (Wollmuth et al., 1998a; Kupper et al., 1998).

Mutations at the NR1 residue Gly618 disturb external Mg^{2+} blockade without affecting block by internal Mg^{2+}. Conversely, mutations made downstream of the N615 site, at NR1 Glu621, NR2A Gln620, or Asn621, appear to influence internal block by Mg^{2+} but have little effect on block by external Mg^{2+} (Kupper et al., 1996, 1998; Wollmuth et al., 1998a,b). These structural data confirm, in part, biophysical results suggesting distinct blocking sites on the basis of different Mg^{2+}-bound dwell times, with unbinding rates differing by more than 100-fold (dissociation from the internally accessible site is faster; Johnson and Ascher, 1990; Li-Smerin and Johnson, 1996). Although mutagenesis data from recombinant receptors suggests that the internal and external Mg^{2+}-blocking sites are physically distinct, mutagenesis has also hinted at complex interactions between the structural determinants of these two binding sites. For example, studies of internal Mg^{2+} block of receptors with substitutions at NR1 Asn616 and NR2 Asn615 suggest that the NR1 subunit plays a larger role in internal Mg^{2+} block than the NR2 subunit. However, single-channel studies show that this difference is due, in part, to effects of internal Mg^{2+} on open probability of these mutant channels, and suggest that NR1 N616S and NR2 N615S mutations reduce internal Mg^{2+} block to a similar extent (Kupper et al., 1998).

C. Internal Polyamine Block of AMPA and Kainate Receptors

Similar to Mg^{2+} block of NMDA channels, calcium-permeable kainate and AMPA receptors are tonically blocked at resting membrane potentials by cytoplasmic polyamine ions (Bowie et al., 1998; Rozov et al., 1998). The initial observations demonstrating that freely diffusible polyamines produce strong voltage-dependent block finally provided a molecular understanding of the complex rectification of native and recombinant calcium-permeable kainate and AMPA receptors, and additionally explained the loss of rectification observed in ex-

cised membrane patches (Bowie and Mayer, 1995; Donevan and Rogawski, 1995; Isa et al., 1995; Kamboj et al., 1995; Koh et al., 1995a; Bowie et al., 1998; Rozov et al., 1998). Bound and unbound polyamines are found in millimolar amounts in virtually all eukaryotic and prokaryotic cells (Watanabe et al., 1991). Most naturally occurring polyamines, such as spermine, spermidine, and putrescine, form complexes with nucleic acids, proteins, and phospholipids, which have implicated them in cell growth and differentiation (Pegg, 1986). More recently, however, the presence of freely diffusible polyamines in the cytoplasm (5–100 μM for spermine and spermidine) (Watanabe et al., 1991) has been shown to exert a profound effect on the gating properties of a number of ion channel families (Nichols and Lopatin, 1997; Williams, 1997a,b; Bowie et al., 1999). In view of the relatively high binding affinity of polyamines for ion channels, this property may, in fact, represent one of their most specific roles in mammalian cells.

Internal polyamine block is not exclusive to non-NMDA receptors, being first identified to account for the "intrinsic" channel gating of inwardly rectifying K^+ channels (Lopatin et al., 1994; Ficker et al., 1994; Fakler et al., 1995) and more recently for neuronal nicotinic acetylcholine receptors (Haghighi and Cooper, 1998). In contrast to K^+ channels and neuronal nicotinic acetylcholine receptors, Mg^{2+} appears to make no significant contribution to rectification of non-NMDA receptors (Bowie and Mayer, 1995). Similar to polyamine block of K^+ channels, the order of potency of naturally occurring polyamines is spermine > spermidine $\gg$ putrescine with dissociation constants calculated at 0 mV of 5 μM, 25 μM, and 1.2 mM, respectively, for homomeric GluR6(Q) channels (Bowie and Mayer, 1995). Recent kinetic studies suggest that the rate of binding by polyamines to the open channel is diffusion-limited and relatively voltage-insensitive, whereas the voltage dependence of equilibrium block is governed largely by the blocker's exit rate from the open channel (Bowie et al., 1998). Furthermore, structure-activity studies suggest that the blocker's residency time in the open channel is not determined exclusively by electrostatic attraction, as might be expected, but is jointly determined by hydrophobic interactions (Cui et al., 1998). Two amino acid residues have, at present, been identified as important structural elements for polyamine binding to the open channel. The first, the Q/R site of the M2 domain, is believed to contribute to the selectivity filter since it determines other permeation properties as described above. The second is a negatively charged aspartate, four amino acid residues downstream of the Q/R site (Dingledine et al., 1992). A recent model proposed by Washburn et al. (1997) suggests that glutamine residues at the Q/R site form a ring of carbonyl oxygens that contribute to or represent the binding site for external Ca^{2+} ions, whereas polyamines interact with the downstream aspartate as well as the Q/R site. This model explains earlier findings that replacement of aspartate with a neutral asparagine of similar size reduced polyamine block without affecting Ca^{2+} permeability (Dingledine et al., 1992).

A major difference between K^+ channels and non-NMDA receptors is the relief of polyamine block observed at extreme positive membrane potentials in the latter case. An early explanation for relief of block was that, at high membrane electric fields, cytoplasmic polyamines permeate non-NMDA receptors, which is observed experimentally as the appearance of outward current flow at very depolarized membrane potentials (Bowie and Mayer, 1995; Koh et al., 1995). This explanation is consistent with estimates of pore dimensions, which are generally accepted to be smaller for K^+ channels (0.3 nm in diameter) (Hille, 1992) than non-NMDA receptors (0.70–0.78 nm diameter) (Burnashev et al., 1996). Experimental evidence directly supporting polyamine permeation was finally obtained by comparing block with polyamines of differing cross-sectional diameter and under experimental conditions where external polyamines were the sole charge carriers (Bähring et al., 1997).

Biochemical estimates of free cytosolic concentrations of polyamines (Watanabe et al., 1991) and knowledge of their affinity as channel blockers (Bowie and Mayer, 1995) suggest that a combination of spermine and spermidine may contribute to channel block. The voltage dependence of putrescine was too weak to account for the block observed in intact HEK293 cells. The ratio of the free concentration of spermine and spermidine in biochemical studies is 1:3 (Watanabe et al., 1991). Using this information with estimates of each blocker's affinity to constrain their fits, Bowie and Mayer (1995) proposed that, from the analysis of the voltage dependence of whole-cell responses, a combination of approximately 50 μM spermine and 150 μM spermidine could account for rectification in intact cells. As yet, it is not known whether the free polyamine concentration is under dynamic regulation although there are extrusion and uptake mechanisms that may be important to consider in this context (Khan et al., 1994).

The recent proposal that polyamines may access both closed and open channels has revealed a novel activity-dependent regulation of calcium-permeable AMPA and kainate receptors that was not considered previously (Bowie et al., 1998; Rozov et al., 1998). Bowie et al. (1998) have proposed that in the closed conformation, kainate receptors possess a water-filled cytoplasm-facing cavity that is accessible to polyamines, an arrangement consistent with the accessibility profile of cysteine-substituted residues on NMDA (Kuner et al., 1996) and AMPA (Kuner et al., 1997) receptors and the structure of a K^+ channel pore (Doyle et al., 1998). Unlike the open state, occupancy of this site has been shown to be insensitive to membrane potential, which may suggest that water molecules in the pore shield

polyamines from the voltage drop across the membrane (Bowie et al., 1998) or that key determinants of polyamine binding lie just beyond the electric field in the closed conformation. Whether the acidic residue four amino acids downstream of the Q/R site in all AMPA and kainate receptor subunits contributes to this polyamine-binding site is unknown. Once in the open state, polyamines are able to "sense" the membrane electric field and re-equilibrate with their binding site in a voltage-dependent manner. The process of re-equilibration is experimentally observed as a voltage-dependent slowing of response rise times by polyamines (Bowie et al., 1998; Rozov et al., 1998). This explanation is favored by the observation that when polyamine affinity is reduced in high permeant ion concentrations, response rise times are faster, reflecting a more rapid re-equilibration of block rate (Bowie et al., 1998).

The relatively slow block of closed AMPA receptors by polyamines accounts for the facilitation of peak responses observed following a train of brief agonist pulses, which may represent a mechanism of short-term plasticity for calcium-permeable non-NMDA receptors (Rozov et al., 1998). A kinetic model of polyamine block also predicts these observations, but additionally suggests that polyamines accelerate channel closure either through an allosteric mechanism or by emptying the pore of permeant ions (Bowie et al., 1998). As yet, it is not known whether activity-dependent modulation of polyamine block fulfills an important role in the CNS. In the hippocampus, for example, basket cells of the dentate gyrus express Ca^{2+}-permeable AMPA receptors that exhibit rapid gating characteristics (Geiger et al. 1995, 1997) believed to be pivotal in defining the functional roles of these interneurons in network oscillatory activity (Jefferys et al., 1996) and feed-back and feed-forward inhibition (Buzsáki and Chrobak, 1995). Although Ca^{2+} permeability and gating properties are undoubtedly important factors in sculpting neuronal behavior, these recent findings suggest that cytoplasmic polyamines may be central to plasticity mechanisms previously thought to be absent in hippocampal and cortical interneurons (McBain, 1998; Bowie et al., 1999).

X. Molecular Composition of the Pore

A. *Outer Vestibules*

As described above, experiments designed to probe the cross-sectional diameter of the permeation pathway concluded that NMDA receptors contain a narrow constriction between broad outer and inner vestibules. Although the molecular nature of the vestibules is unknown, recent reports suggest that the outer vestibule contains a divalent ion-binding site that occupies a shallow position within the electric field (Jahr and Stevens, 1993; Paoletti et al., 1995; Premkumar and Auerbach, 1996; Sharma and Stevens, 1996b). Similarly, studies of polyamine block suggest that polyamines may occupy an inner vestibule before gating (Bowie et al., 1998). Thus, both vestibules may serve unique physiological functions that may control glutamate receptor function.

B. *Narrow Constriction and Selectivity Filter*

Do residues implicated by mutagenesis studies in permeation and block actually present side chains to a presumably water-filled pore? Residues within the M2 region of both NR1 and NR2 of the NMDA receptor appear to be accessible to covalent labeling by reactive sulfur-containing ions (Kuner et al., 1996, 1997). Particularly interesting is the finding that residue NR1 Asn616 (the Q/R/N site) and equivalent positions in NR2 subunits are sensitive to channel-blocking thiol-reactive compounds applied to both the extra- and intracellular sides of the channel in the presence of glutamate and glycine. This NR1 residue has been suggested to be at the hairpin turn of the re-entrant M2 loop that lines the channel lumen on the basis of the observation that larger thiol-reactive compounds can modify residues on either side of this loop when applied internally, whereas NR1 Asn616 (and equivalent positions in NR2) are the only residues reactive toward these compounds when applied from the extracellular surface. This suggestion is supported by mutagenesis data suggesting that NR1 Asn616 and Asn615 help to form the narrowest portion of the pore (Wollmuth et al., 1996, 1998a). Moreover, other positions such as a hydrophilic stretch of amino acids on the downstream side of Asn616 are reactive to cysteine-modifying reagents, suggesting that these residues might comprise the selectivity filter and narrow pore constriction (Kuner et al., 1996; Fig. 6). Wollmuth et al. (1998a) have argued that NR2 Asn residues form the selectivity filter that discriminates external Mg^{2+} from other ions, whereas NR1 Asn616 largely acts to limit channel diameter (Wollmuth et al., 1996, 1998a; Kuner et al., 1996; Williams et al., 1998). As expected, pore-enlarging mutations in either subunit render even more residues reactive with pore-blocking cysteine-modifying reagents (Kuner et al., 1996), but unexpectedly do not alter external Mg^{2+} blockade or permeability. Thus, a central point that has emerged in recent years is the asymmetrical contribution of NR1 and NR2A residues to 1) pore diameter, 2) external Mg^{2+} block, and 3) Ca^{2+} permeability. Such asymmetry may be mirrored by AMPA and perhaps kainate receptors in which varying proportions of different subunits can be incorporated. Thus, functional results from studies of mutant and differentially edited receptors as well as biochemical data in the form of covalent-labeling experiments that suggest M2 residues reside in the pore. Moreover, the many parallels between the effects of the Q/R/N site and nearby residues in NMDA receptors and polyamine block and Ca^{2+} permeability of non-NMDA receptors suggest that the both classes of receptors might share similar pores.

XI. Association of Glutamate Receptors with Intracellular Proteins

A variety of intracellular proteins that bind to glutamate receptors have just recently been described. They appear to be structurally and functionally quite important not only for receptor targeting or clustering, but also for modulation of receptor activity and activation of signaling pathways. Moreover, receptor targeting and clustering is regulated during development (Rao et al., 1998), depends on synaptic activity, and might even play a role in LTP. For example, tetanic stimulation of hippocampal slice cultures induced long-lasting AMPA receptor clustering observed by introduction of recombinant GluR1 tagged with green fluorescent protein (Shi et al., 1998).

The yeast two-hybrid system was instrumental in the initial identification of several glutamate receptor-associated proteins, including proteins containing PDZ domains [e.g., proteins of the PSD-95 family, GRIP, AMPA receptor-binding protein (ABP)]. Some PDZ domains, which are 90-amino acid repeats that are known to be involved in protein-protein interactions, associate with the C termini of certain AMPA and NMDA receptor subunits. Coimmunoprecipitation confirmed the association of glutamate receptors with PDZ domain-containing proteins and signaling molecules (Src, calmodulin, G proteins). Some of these proteins compete for binding to the receptor (often dependent on the calcium concentration) and in some cases binding can be regulated by phosphorylation.

A. AMPA Receptors

The first ABP cloned was GRIP. GRIP contains seven PDZ domains and interacts through its fourth and fifth PDZ domains with the C-terminal motif SVKI* (*denotes a stop codon) of GluR2, GluR3 and possibly GluR4c (Dong et al., 1997). At least two other synaptic proteins interact with the C-tails of these subunits, Pick1 (Xia et al., 1998) and ABP (Srivastava et al., 1998). Like GRIP, ABP binds to the very C terminus of GluR2/3, and both proteins can form homo- and heteromultimers through PDZ-PDZ domain interactions. However, GRIP and ABP do not interact with PSD-95, another PDZ domain protein that binds to NMDA receptors (Srivastava et al., 1998). Thus, the PDZ domain-containing proteins that bind AMPA and NMDA receptors might form distinct complexes. The binding of the C-tail of GluR2/3/4c to Pick1 may be involved in the clustering of AMPA receptors. In fibroblasts cotransfected with GluR2 and Pick1, Pick1 induced clustering (Xia et al., 1998). That the GluR2 C-tail is important for receptor clustering is confirmed by the observation that injection of GluR2 C-tail peptides, which compete with the binding to Pick1 or other proteins, disrupted AMPA receptor clustering in cultured hippocampal neurons (Dong et al., 1997).

GluR2 and GluR4c, but not GluR1, GluR3 and GluR4, specifically interact with an *N*-ethylmaleimide-sensitive fusion protein (NSF) but not other chaperone-like proteins (Osten et al., 1998; Nishimune et al., 1998; Song et al., 1998). The homohexameric NSF is an ATPase involved in various membrane fusion events, such as inter-Golgi protein transport and exocytosis of synaptic vesicles. The synaptic NSF attachment proteins (SNAPs) are an essential component for the latter. The NSF binding site is distinct from the GRIP/ABP-binding site and resides in the cytoplasmic C-tail of GluR2 and GluR4c at about 20 to 10 amino acids from the very C-terminal amino acid. The interaction appears to occur with three residues (Lys844, Gln853, and Asn851 in rat GluR2) that are only found in GluR2 and GluR4c (Nishimune et al., 1998). GluR2, NSF, and SNAP were coimmunoprecipitated and colocalized in dendrites and axonal shafts using immunohistochemistry (Osten et al., 1998; Song et al., 1998). One NSF hexamer seems to associate with one AMPA receptor. The binding of GluR2-NSF to SNAP was ATP-dependent (Osten et al., 1998). Intracellular perfusion with synthetic peptides that compete for the NSF-binding site on AMPA receptors decreased the amplitudes of miniature EPSCs in cultured hippocampal neurons (Song et al., 1998) and AMPA-mediated EPSCs in CA1 neurons in slices within minutes (Nishimune et al., 1998). Similar results were obtained with anti-NSF antibodies in CA1 neurons, which suggeststhat postsynaptic AMPA receptor function can be regulated rapidly by NSF. From these results it is proposed that rapid (minutes to less than 1 h) AMPA receptor turnover occurs at the synaptic membrane and that NSF acts as a chaperone for (re)insertion of new or recycled AMPA receptors into the plasma membrane. In addition, F-actin plays a role in localizing AMPA receptor clusters to synapses and in some neurons also in the clustering of AMPA receptors. In cultured hippocampal GABAergic neurons, disruption of F-actin by latrunculin A disturbed the synaptic localization but not the clustering of GluR1-containing receptors (Allison et al., 1998). In contrast, in pyramidal neurons, both synaptic localization and clustering were partially reduced by latrunculin A. However, it is not known how AMPA receptors associate with actin. Finally, the A kinase-anchoring protein AKAP-79 functions as a signaling scaffold for PKA, PKC, and calcineurin at postsynaptic densities in neurons. Calcineurin is inactive when bound to AKAP, and anchoring of PKA to AKAPs seems to be required for the modulation of AMPA receptors (Rosenmund et al., 1994).

A new concept is the recently reported regulation of AMPA receptors by G proteins (Wang et al., 1997). In cultured cortical neurons and in membrane preparations, AMPA inhibited both the ADP ribosylation of $G\alpha_{i1}$ induced by pertussis toxin and forskolin-induced cAMP elevations. Both of these effects were blocked by CNQX. This demonstrates that G_i proteins were activated by

AMPA, a compound that is thought to be inactive on all metabotropic glutamate receptors. $G\alpha_{i1}$ could be coimmunoprecipitated with GluR1 by an anti-GluR1 antibody in AMPA-treated cultures but not in control cultures, suggesting an involvement of ionotropic AMPA receptors in the metabotropic signaling cascades. The mechanism of interaction between AMPA receptors and G proteins is still unclear, although an adaptor protein might be involved. In addition, AMPA application to cultured cortical neurons activated mitogen-activated protein (MAP) kinase, which could be inhibited by pertussin toxin or by lack of extracellular calcium (Wang and Durkin, 1995). The calcium entry through Ca^{2+}-permeable AMPA receptors may be responsible for the MAP kinase activation. Furthermore, AMPA induced the association of the G protein β subunit with the Ras complex, Raf kinase, and MEK1. This could result if AMPA receptor activation induced the release of $G\beta\gamma$ subunits from $G\alpha$, which in turn activates the Ras protein complex and subsequently MAP kinase. It is becoming clear from all of these findings that AMPA receptors are embedded within a complex protein network, which points to a more complicated role than simple ion transport.

B. NMDA Receptors: Signaling Molecules and Proteins Lacking PDZ Domains

A variety of signaling proteins, actin binding, and filamentous proteins can also bind to NR1, NR2A, or NR2B subunits. Src was found to be associated with NMDA receptors by coimmunoprecipitation (Yu et al., 1997; see above), tyrosine-phosphorylated NR2A and NR2B bind to the src homology domain region 2 (SH2) of phospholipase Cγ in vitro (Gurd and Bissoon, 1997), and autophosphorylated CAMKII has a high affinity for the NR2B but not NR2A C-tail (Strack and Colbran, 1998). The association between these signaling molecules suggests functional coupling. Indeed, Src can phosphorylate NMDA receptors and enhance their activity (see above). Whether phospholipase C is functionally coupled to NMDA receptors is to our knowledge unknown. However, the phosphorylation of AMPA receptors by autophosphorylated CAMKII targeted to the PSD by its interaction with the C-terminal 49 amino acids of NR2B is thought to increase synaptic strength during LTP (see above).

α-Actinin is an actin-bundling protein that binds not only to NR1 but also to the NR2B C terminus and may play a role in localization of the NMDA receptor (Wyszynski et al., 1997). Depolymerization of F-actin by latrunculin A completely dispersed α-actinin and reduced the number of synaptic NR1 clusters by 60% in cultured hippocampal neurons (Allison et al., 1998). The NMDA receptor clustered together with PSD-95 appeared to relocate to the cell bodies. In whole-cell recordings actin depolymerization induced by calcium, but not destabilization of microtubuli by colchicine, reduced NMDA receptor activity (Rosenmund and Westbrook, 1993). Furthermore, the reduced calcium-dependent depolymerization of actin in hippocampal neurons of gelsolin knockout mice was associated with reduced current rundown of NMDA receptors (Furukawa et al., 1997). However, the finding that NMDA receptors in patches from cultured mouse neurons are mechanosensitive (Paoletti and Ascher, 1994) probably has a different explanation than an association between NMDA receptors and cytoskeletal proteins, because lysophospholipids that are thought to alter lipid packaging in membranes mimicked the effect (Casado and Ascher, 1997).

Moreover, in biochemical experiments the NR1, NR2A, and NR2B subunits interacted with the actin-binding protein spectrin (Wechsler and Teichberg, 1998). Spectrin seems to bind to different sites of the NR2B C terminus than actinin or PSD-95. The association between NR2B and spectrin can be weakened by calcium and phosphorylation by the Fyn kinase. In contrast, the spectrin interaction with NR1 can be antagonized by calcium/calmodulin or by phosphorylation of NR1 by PKA or PKC. The functional consequences of the spectrin assocation are not yet known.

Depending on the calcium concentration, calmodulin can bind to two different sites in the NR1 C terminus. Calcium/calmodulin seems to bind with high affinity close to the serines in the C1 cassette, and PKC activation can weaken this interaction (Hisatsune et al., 1997). The lower affinity binding site also binds α-actinin 2 and is found in all NR1 splice variants in the C0 cassette that precedes C1. Calcium-dependent binding of calmodulin to this site seems to mediate calcium-dependent inactivation of NMDA receptors (Zhang et al., 1998), possibly via calcium/calmodulin-dependent release from the cytoskeleton (e.g., from α-actinin). At the single-channel level calcium-dependent calmodulin binding to NR1 reduced the open probability of NMDA receptors by up to 4- to 5-fold (Ehlers et al., 1996; Hisatsune et al., 1997). The C1 cassette of NR1 not only binds calmodulin but also the protein yotiao, which is expressed in muscle, pancreas, and brain (Lin et al., 1998). The filamentous protein can be coimmunoprecipitated with NR1 in brain. Yotiao is found in the PSD fraction, is somato-dendritically localized, and colocalizes with NR1. In addition, the NR1 C1 cassette interacts with the ends of the 68-kDa neurofilament subunit NF-L, which is colocalized in dendrites and cofractionates with NR1 (Ehlers et al., 1998). Furthermore, in quail fibroblasts, this cassette was found to be crucial for the formation of receptor-rich domains (Ehlers et al., 1995). Interestingly, the clustering was disprupted by PKC activation, which was dependent on Ser890 in the C1 exon (Tingley et al., 1997). In summary, NMDA receptor activation might regulate its own activity, its association with intracellular proteins, and its clustering.

C. NMDA Receptors: PDZ Domain-Containing Proteins

In addition to the actin system, other molecules containing PDZ domains form complex networks with NMDA receptors and possibly the tubulin system, which seems to play an important role for the localization, clustering, and function of NMDA receptors. A variety of proteins from the PSD-95 family named after their first discovered member, PSD-95, can bind to the C termini of the NMDA receptor subunits. Using the yeast two-hybrid system and the NR2A C-tail as bait, Kornau et al. (1995) identified PSD-95 as an NR2A-interacting protein (reviewed by Kennedy, 1997). The PSD-95 family proteins are also called channel-associated proteins of synapses (chapsyns) and consist of at least four proteins: SAP90/PSD-95, SAP97, PSD-93/chapsyn-110, and SAP102 (Kennedy, 1997). Each chapsyn contains three PDZ domains, one src homology domain 3 (SH3) and one guanylate kinase (GK) domain. The SH3 and GK domains as well as the PSD domain can mediate protein-protein interaction. The GK domain has no kinase activity, but can bind GMP. Among the three PDZ domains of PSD-95, the second has the highest affinity for the E(T/S)XV* motif in the C termini of all four NR2 subunits as well as the NR1-3 and NR1-4 splice forms (which bear the C2′ terminus). PSD-95 is an abundant cytoskeletal protein, found in the postsynaptic fraction of synaptosomes, but it also occurs presynaptically. The C termini of NR2A, B, and C can also interact with PSD-93/chapsyn-110, SAP97, and SAP102 (Müller et al., 1995, 1996; Kim et al., 1996; Niethammer et al., 1996). All of these proteins have also been found associated with shaker-type K channel subunits and the plasma membrane Ca^{2+} ATPase isoform 4b, which have similar C-terminal motifs (Kim et al., 1998). In heterologous cells, chapsyn-110 can heteromultimerize with PSD-95 to promote clustering of NMDA receptors and Kv1.4 potassium channels, which are otherwise diffusely distributed (Kim et al., 1996). SAP97 is found predominantly in axons and in glutamatergic terminals (Müller et al., 1995). The postsynaptic protein SAP102 was coimmunoprecipitated with NR1 subunits by anti-NR1 antibodies, and binds also to the very C termini of recombinant NR2A and 2B and the cortical cytoskeleton (Müller et al., 1996; Lau et al., 1996). Another protein containing a PDZ domain but with an "inverse" structure is the synaptic scaffolding molecule (S-SCAM; Hirao et al., 1998). S-SCAM contains five C- but not N-terminal PDZ domains which interact with NMDA receptors and neuroligins, and in addition the N- but not C-terminal GK-like domain binds to SAP90/PSD-95-associated proteins (see below).

The PDZ domain contain proteins and others such as actinin and spectrin may provide a scaffold for the localization of signaling proteins to position them close together. For example, anti-PSD-95 antibodies coimmunoprecipitated NR1, NR2B, and α-actinin (Wyszynski et al., 1997). Moreover, neuronal nitric acid synthase can bind to PSD-95 via a PDZ-PDZ interaction and could, thus, be localized close to NMDA receptors (Brenman et al., 1996). Recently, a brain-specific synaptic Ras-GTPase-activating protein, synGAP, was cloned that binds to PSD-95 (Kim et al., 1998; Chen et al., 1998). SynGAP coimmunoprecipated and colocalized with NMDA receptor subunits and PSD-95 and is enriched at excitatory synapses. In vitro, synGAP stimulated the GTPase activity of Ras, suggesting that it might be a negative regulator of Ras at certain synapses (Kim et al., 1998). However, the Ras-GTPase-activating activity of synGAP can be inhibited by phosphorylation by CAMKII, which would allow the activation of the MAP kinase pathway by Ras after NMDA receptor activation (Chen et al., 1998).

Different members of the PSD-95 family and S-SCAM can bind to several proteins that may function as scaffold proteins, e.g., neuroligins (Irie et al., 1997), cysteine-rich interactor of PDZ three (CRIPT; Niethammer et al., 1998), microtubule-associated protein 1A (MAP1A; Brenman et al., 1998), the different isoforms of guanylate kinase-associated proteins (GKAPs; Kim et al., 1997; Kawashima et al., 1997), and the SAP90/PSD-95-associated proteins (Takeuchi et al., 1997). Neuroligins are membrane-spanning cell adhesion molecules that interact with β-neurexins and form intercellular junctions. Neuroligins bind to the third PDZ domain of PSD-95 family proteins, which then could recruit NMDA receptors that have high affinity for the second PDZ-domain. The third PDZ domain of PSD-95 can also associate with CRIPT, which could link to the tubulin system, since CRIPT coimmunoprecipitated with PSD-95 and tubulin (Niethammer et al., 1998). In COS-7 cells, CRIPT reorganized the microtubuli to thick fibers and recruited PSD-95 to the microtubuli. Another link to the tubulin system may occur via the GK domain of PSD-93/chapsyn-110, which can interact with MAP1A (Brenman et al., 1998). MAP1A regulates microtubule dynamics.

The GKAPs were originally isolated using the yeast two-hybrid system with the GK domain of PSD-95 as bait. They can interact with the GK domain of SAP97, SAP102, and chapsyn-110 and are enriched in the PSDs (Kim et al., 1997; Kawashima et al., 1997). Furthermore, in cultured hippocampal neurons, the postsynaptic GKAP seems to be specifically expressed at glutamatergic but not GABAergic synapses (Naisbitt et al., 1997). The functions of GKAP are not clear, but the evidence points to an association with cytoskeletal proteins. During the development of synapses in cultured hippocampal neurons, PSD-95 and GKAPs are clustered at presumptive postsynaptic sites opposite presynaptic terminals before NMDA and AMPA receptors appear at these sites (Rao et al., 1998). Thus, it seems that the scaffold is provided before the postsynaptic receptors cluster.

Taken together, these findings point to an informationally rich cluster of signaling proteins that target glutamate receptors and probably their associated kinases and phosphatases to subsynaptic membranes. NMDA receptors are clustered by chapsyns and neurofilaments and are localized within the cell by actin filaments. Moreover, NMDA receptors not only directly bind to signaling molecules (calmodulin, CAMKII) but in addition are indirectly complexed in combination with other signaling molecules (K^+ channels and possibly Ca^{2+} pumps, and the Na^+ channel α subunit) via interaction with actin-binding and PDZ domain-containing proteins.

D. Kainate Receptors

The functional regulation of kainate receptors by association with intracellular proteins is just being uncovered. Members of the PSD-95 family (PSD-95/SAP90, SAP97, and SAP102) colocalize and associate with some kainate receptor subunits (Garcia et al., 1998). The interaction between kainate receptor subunits and SAP90 occurs via SAP90's PDZ1 domain and the GluR6 C terminus (ETMA*) and via SAP90's SH and GK domains and KA2. SAP90 clusters GluR6 and KA2 subunits when heterologously expressed. Moreover, when SAP90 was coexpressed with GluR6/KA2 or GluR6 homomers, receptor desensitization was reduced, attesting to a functional role of PDZ domain-containing proteins (Garcia et al., 1998).

XII. Genetic Regulation of Receptor Expression

The level of expression of each glutamate receptor subunit is determined at any particular time by the balance of the rates of gene transcription, mRNA translation, mRNA degradation, and protein degradation; other processes such as receptor assembly and synaptic targeting mechanisms provide additional controls over the appearance of functional receptors where they are needed. Glutamate receptor expression is not static but varies in a cell-specific manner throughout development and in response to trauma and other environmental factors. Examples are the developmental switch from NR2B to NR2C subunits in cerebellar granule cells about 2 weeks after birth (Watanabe et al., 1992), the altered expression of different AMPA receptor subunits after ischemia or seizures (Pollard et al., 1993; Pellegrini-Giampietro et al., 1994; Prince et al., 1995; Ying et al., 1997; Gorter et al., 1997), and the induction of GluR1 expression during chronic exposure to morphine or other drugs of abuse (Fitzgerald et al., 1996). The injection into the ventral tegmental area of a GluR1-expressing herpes simplex vector sensitizes rats to certain behavioral effects of morphine (Carlezon et al., 1997), pointing to a functional role for GluR1 up-regulation by opioids. The relative abundance of GluR2 among other AMPA receptor subunits differentially affects Ca^{2+} permeability and rectification, as one consequence of the variable rather than fixed number of GluR2 subunits in an AMPA receptor (Washburn et al., 1997). All of these findings indicate that the mechanisms controlling the expression of glutamate receptor subunits are important regulatory determinants of receptor function. Below we provide a brief discussion of progress made in the past few years on the transcriptional and translational control of subunit expression. A more detailed review, including a discussion of insights from genetically modified animals, appears elsewhere (Myers et al., 1999).

A. Transcriptional Control

To date the 5′-flanking regions containing the proximal promoter have been cloned and preliminarily characterized for the following genes: *NR1* (Bai and Kusiak, 1995; Bai et al., 1998), *NR2B* (Sasner and Buonnano, 1996; Klein et al., 1998), *NR2C* (Suchanek et al., 1995, 1997), *GluR1* (Borges and Dingledine., 1998), *GluR2* (Köhler et al., 1994; Myers et al., 1998), and *KA2* (Huang and Gallo, 1997). The glutamate receptor genes characterized to date have several features in common. Promoters in all of these genes appear to be GC-rich, to lack TATA/CAAT boxes, and to have multiple transcriptional start sites. Regulatory elements have been identified both up- and downstream of the principle transcriptional start site in several genes (Suchanek et al., 1997; Huang and Gallo, 1997; Myers et al., 1998), and one expects this to be true for most of these genes. The Sp1 regulatory element as well as neuron-restrictive silencer elements appear to be present in most, if not all, of these promoters.

We are ultimately interested in understanding the mechanisms by which gene expression responds to environmental signals within an individual neuron. However, most laboratories have focused initially on understanding the basis of neuron-specific expression. For example, GluR2 is expressed nearly exclusively in neurons in the brain, and this pattern is recapitulated in cultured cortical neurons transfected with a luciferase reporter plasmid driven by a segment of the GluR2 promoter sequence as short as 250 bp (Myers et al., 1998). A combination of gel shift and mutation analysis, and cotransfection with plasmids encoding putative transcription factors, identified three functional regulatory elements in the *GluR2* gene, Sp1, NRF-1, and the neuron-restrictive silencer RE1/NRSE; a 25- to 32-bp sequence was also identified that did not itself contain any regulatory elements but instead served as an important spacer or bridge sequence between the Sp1 and NRF-1 elements. The RE1/NRSE sequence that acts in some other genes as a powerful silencer in non-neuronal cells (Kraner et al., 1992) served only as a fine-tuning modulator of expression in the GluR2 promoter. Moreover, careful mutation analysis of the entire sequence showed that no single regulatory element was required for neuron-specific expression of the GluR2 gene in rat cortical and glial cultures (Myers et al., 1998). Thus, the basis

for neuron-specific GluR2 expression remains to be found (see Myers et al., 1998 for speculation on possible mechanisms).

Similar to the situation with the *GluR2* gene, the RE1/NRSE silencer makes only a minor contribution to cell-type specific expression of the *NR1* gene (Bai et al., 1998). Elements that recognize two single-stranded DNA-binding proteins were also identified in the *NR1* gene (Bai et al., 1998), but their role in neuron-specific expression is unknown. Sasner and Buonnano (1996) described an 800-bp region of the NR2B promoter that was sufficient to limit reporter expression to neurons in transgenic mice, and deletion analysis was later used to identify a 150-bp region surrounding the major transcriptional start site that confers neuron-specific expression of NR2B (M. Sasner and A. Buonnano, submitted). Again, however, the mechanism of neuron selectivity has not yet been established. Likewise, neuron-selective expression is conferred by promoter sequences for *KA2* (Huang and Gallo, 1997) and *NR2C* genes (Suchanek et al., 1997), but the regulatory elements responsible and the mechanisms conferring cell-type specificity on these promoters are yet to be identified.

B. Translational Control

The 5′ UTR of many of the glutamate receptor mRNAs is unusually long, for example, at least 282 bases for NR2A (Meguro et al., 1992; full-length mRNA may be longer), up to 481 bases for GluR2 (Myers et al., 1998), and up to 772 bases for NR2C (Suchanek et al., 1995) depending on the transcriptional start site. The exceptionally long 5′ UTR of NR2B (up to 1199 base pairs) is broken up by three introns (Klein et al., 1998). These long 5′ UTRs often exhibit stetches of high GC content and sometimes contain multiple out-of-frame AUG codons that could act as decoys for scanning ribosomes. There are up to five upstream AUGs in the GluR2 mRNA (Myers et al., 1998), and 12 in NR2B (Klein et al., 1998). In contrast, only 10% of eukaryotic 5′ leader sequences contain any AUG codons (Kozak, 1991; note that the 772 base 5′UTR of NR2C has no AUG codons). Translational suppression has been inferred for NR1 mRNA natively expressed in PC12 cells because no trace of NR1 protein can be detected in these cells despite a moderately high mRNA level (Sucher et al., 1993). However, translational supression mediated by the 5′UTR has so far been demonstrated only for the *NR2A* and *GluR2* genes.

Wood et al. (1996) used a combination of in vitro translation in rabbit reticulocyte lysate and the *Xenopus* oocyte expression system to study the translatability of NR2A mRNAs possessing different 5′ UTRs. They found that removal of most of the 282 bases of 5′ UTR increased electrophysiological responses mediated by NR1/NR2A receptors by over 100-fold. Removal of each individual upstream AUG only minimally increased translation, whereas mutations within a 15-base sequence of the 5′ UTR that disrupted a proposed stem-loop structure relieved translation inhibition considerably. A similar situation may hold with GluR2 translation. Myers et al. (1998) found that the 5′UTR of GluR2 mediated a 30- to 60-fold translational suppression in *Xenopus* oocytes and an in vitro rabbit reticulocyte translation system. The upstream AUG's played a minor role, but a broad region near the 5′ end of the mRNA that harbored a repeat sequence appeared to mediate much of the translational inhibition. Interestingly, transcriptional start sites straddled this control region, such that some but not all GluR2 mRNAs would be expected to be translationally suppressed. This finding highlights the need for cautious interpretation of methods (in situ hybridization, single-cell RT-PCR) designed to infer subunit distribution based on mRNA localization.

From these initial studies, it is clear that regulation of at least the NR2A and GluR2 subunits can occur at the translational as well as transcriptional level. It will be important to understand the conditions under which such translational control is actually realized in neurons, because constitutive suppression would make little biological sense.

XIII. Therapeutic Potential: Clinical Trials

It is well known that glutamate and aspartate can be neurotoxins, especially when energy supply is compromised (Greene and Greenamyre, 1996; Choi, 1998). This has given rise to the proposal that neurodegeneration associated with a variety of acute and chronic disorders (ischemic stroke, epilepsy, Parkinson's Disease, AIDS dementia, among others) may be caused in part by overactivation of glutamate receptors. Indeed, there is evidence from animal studies for marked neuroprotective effects of NMDA and AMPA receptor antagonists in models of ischemic stroke, epilepsy, and Parkinsonism. There is also growing support for the proposal that chronic pain can be relieved by antagonists of NMDA and perhaps kainate receptors (see below). Finally, there are suggestions that slowing AMPA receptor desensitization may have a cognitive enhancing effect. Rather than summarizing a large number of animal studies, we intend to review primarily evidence from clinical trials testing the use of drugs targeted to glutamate receptors in humans. The NMDA receptor antagonists are further along in clinical studies of stroke, Parkinson's disease, and pain than are the AMPA or kainate receptor antagonists.

Epilepsy has been considered a potential therapeutic target for glutamate receptor antagonists. Indeed, the common anticonvulsant valproate, in therapeutic concentrations, has been shown recently to bind to AMPA receptors in the human hippocampus (Künig et al., 1998). Thus valproate may act as an anticonvulsant partly by blocking AMPA receptors. However, epilepsy as a target, although attractive from a mechanistic point

of view, has waned in recent years with the introduction in the mid-1990s of five new anticonvulsants, and the natural reluctance of physicians to expose their patients to the possibility of an unexpected seizure with untested drugs. To our knowledge, there are no ongoing clinical trials of glutamate receptor antagonists for any of the epilepsies.

A. Ischemic Stroke

Stroke was the first clinical indication considered for glutamate receptor antagonists. Aside from the considerable challenges involved in crafting a drug that has appropriate pharmacokinetics and bioavailability, there are at least three additional hurdles to be overcome to develop a useful glutamate receptor antagonist for ischemic stroke. First, Grotta (1995) and Lees (1997) point out the extreme heterogeneity of human stroke patients, and the long delay until treatment compared with animal studies; they question whether the relatively homogenous animal models can provide information with good predictive value. They also point out the usefulness of being able to predict which stroke victims would show the strongest responses to glutamate receptor antagonists. Second, drug concentrations high enough to be neuroprotective typically block glutamate receptors in healthy brain tissue, which makes it very difficult to achieve a satisfactory side effect profile. Cardiovascular side effects (hypo- and hypertension) have been the most prominent and dose-limiting in many small-scale human studies. To solve this problem, one must either identify particular receptor subtypes that contribute to neuron damage but play little role in healthy brain or develop a drug that blocks receptors only in the ischemic region (i.e., context-dependent block). The recent demonstration that the potency of some ifenprodil analogs is increased at the acidic pHs typical of ischemic tissue (Pahk and Williams, 1997; Whittemore et al., 1997; Mott et al., 1998) points the way to optimizing the ifenprodil structure to maximize effects at acidic pH values that occur during ischemia. Third, the earlier the drug is administered the more protection one sees in animal models; therefore, one would like to identify a drug that can be taken prophylactically for those at risk for stroke. Along these lines, it may be possible to develop an ifenprodil analog with a large enough potency boost at low pH that at therapeutic concentrations the drug is inactive at normal pH (Mott et al., 1998). Low-affinity uncompetitive blockers may offer an alternative approach, because strong block would result during periods of intense activation, but recovery from block might be fast enough to have minimal impact on normal synaptic transmission.

To date, this constellation of features is not found in any individual drug, but a number of NMDA receptor antagonists have been subjected to early clinical trials for stroke. These include the uncompetitive channel blocker aptiganel (Cerestat, CNS1102), the competitive glutamate site blocker selfotel (CGS 19755), the competitive glycine site antagonist ACEA 1021, and the ifenprodil analog eliprodil. The selfotel trial was terminated early due to lack of efficacy at tolerable doses (Davis et al., 1997), as was the eliprodil trial (reported in Lees, 1997). In the case of eliprodil (an ifenprodil analog), the drug is known to block N-, P-, and Q-type Ca^{2+} channels at doses that also block NMDA receptors (Bath et al., 1996; Biton et al., 1997); this secondary effect could be dose-limiting. Thus, the results to date have not been especially encouraging, but the marked therapeutic effects of NMDA receptor antagonists in animal models of focal ischemia gives rise to continued optimism. Phase III trials with magnesium sulfate and aptiganel are currently in progress.

Early stroke trials with the AMPA receptor antagonist NBQX were discontinued due to the insolubility of the drug, which precipitated in kidneys causing necrosis. Second-generation AMPA receptor antagonists that are much more water soluble are under development in several companies. For example, the novel water-soluble AMPA receptor antagonist, YM872, is neuroprotective in several in vitro assays (Small et al., 1998). Likewise, introduction of a methylphosphonate group into a quinoxalinedione produces a water-soluble AMPA receptor antagonist (ZK200775) which, after systemic administration in rats, appears to exhibit an unusually long therapeutic window (>4 h) for neuroprotection following occlusion of the middle cerebral artery (Turski et al., 1998).

B. Neuropathic Pain

Chronic pain, such as that due to injury of peripheral or central nerves, has often proved very difficult to treat, even with opioids. An early study concluded that chronic pain in patients associated with spinal cord injury could be markedly reduced by very low-dose ketamine (Eide et al., 1995). Subsequently several case reports indicated that systemic administration of amantadine or ketamine could substantially reduce the intensity of trauma-induced neuropathic pain, in some cases eliminating the pain altogether (e.g., Eisenberg and Pud, 1998). Small-scale double-blind, randomized clinical trials corroborated that amantadine could significantly reduce neuropathic pain in cancer patients (Pud et al., 1998), and ketamine could reduce pain in patients with peripheral nerve injury (Felsby et al., 1996), peripheral vascular disease (Persson et al., 1998), or kidney donors (Stubhaug et al., 1997). "Wind-up pain" produced by repeated pinpricking was also dramatically reduced. These findings suggest that central sensitization caused by nociceptive inputs can be prevented by these drugs. Local infiltration of ketamine (5 ml of 5.3 mM) produced transient relief from pain associated with first- or second-degree burns, but this was ascribed to a local anesthetic action (Pedersen et al., 1998). Wiesenfeld-Hallin (1998) summarized clinical studies that indicate ketamine can

also reduce the need for opiates in the treatment of severe pain. Most of these findings are very encouraging but suffer from the problem of demonstrating that the therapeutic effects of ketamine and amantadine were indeed mediated by block of NMDA receptors. In this regard, Mathisen et al. (1995) showed that chronic neuropathic orofacial pain could be relieved transiently by racemic ketamine and its two stereoisomers, with effective serum concentrations of the three drugs related to their affinity for the NMDA receptor. It will be important to examine more specific NMDA receptor antagonists to evaluate adverse effects of these drugs more carefully and to confirm these findings with larger scale clinical trials.

One potential problem with administering NMDA antagonists chronically to treat chronic pain is the impairment in memory and attention that may result (e.g., Malhotra et al., 1996). NMDA receptor antagonists such as ketamine can also produce a psychotic state in humans reminiscent of schizophrenic symptoms (Krystal et al., 1994). These considerations have encouraged investigators to explore other molecular targets. The prominence in dorsal root ganglion cells of both functional kainate receptors (Huettner, 1990) and the GluR5 subunit (Partin et al., 1993; Bahn et al., 1994) suggests the possibility of targeting kainate receptors for chronic pain. Several biotechnical and pharmaceutical companies are currently testing kainate receptor antagonists in animal models of chronic pain. For example, Simmons et al. (1998) showed that a selective GluR5 antagonist, LY382884, an isoquinoline derivative that has very low affinity for all AMPA receptors as well as for GluR6, exhibited analgesic actions in formalin-injected rats. Intraperitonial doses of LY382884 that were analgesic had no detectable ataxic effects. These results are encouraging, and one anticipates a rush of similar reports in the upcoming year.

C. Parkinson's Disease

There is ample rationale from animal studies to suspect that glutamate receptor antagonists might be beneficial in Parkinson's disease (Blandini and Greenamyre, 1998). The anti-Parkinsonian drug, amantadine, is now known to be an NMDA receptor channel blocker (Blanpied et al., 1997). Amantadine is seldom used alone due to limited efficacy. However, a small-scale clinical trial demonstrated the value of amantadine as add-on therapy with L-dopa. Amantadine reduced the severity of dyskinesias by 60% in these patients without reducing the antiparkinsonian effect of L-dopa itself (Verhagen Metman et al., 1998). It is not clear, however, whether the therapeutic mechanism involves NMDA receptor blockade or some other action of the drug. Other small-scale clinical trials with ifenprodil or dextromethorphan, both NMDA receptor blockers, were mildly supportive (Metman et al., 1998) or inconclusive (Montastruc et al., 1997).

D. Cognitive Enhancement

Both NMDA and AMPA receptors play critical roles in learning and some forms of associative memory in animals (e.g., Tsien et al., 1996). Could drugs that facilitate glutamate receptor transmission enhance certain forms of cognition in humans? One such compound under current study is the benzoylpiperidine CX516, which produces a mild relief from desensitization in AMPA receptors (Arai et al., 1996). In one double-blind, placebo-controlled study, CX516 produced a dose-dependent improvement in the ability to recall nonsense syllables for 30 elderly individuals (Lynch et al., 1997). Similarly, CX516 was reported to improve memory scores in four other placebo-controlled, double-blind tests in a small sample of young individuals (Ingvar et al., 1997). In a multicenter, placebo-controlled, double-blind randomized study of 91 Alzheimer's patients, a 10-week regimen of D-cycloserine, a partial agonist at the glycine recognition site of NMDA receptors, appeared to improve recall in an implicit memory test (Schwartz et al., 1996). How prolongation or enhancement of excitatory synaptic currents throughout the brain could lead to a selective enhancement of memory rather than an increase in "synaptic noise" that might instead be disruptive or perhaps even neurotoxic is unclear, at least to us. Nonetheless, the initial reports are reasonably encouraging; larger scale clinical trials and studies that demonstrate the mechanism of action of these drugs for improving memory would be very interesting.

XIV. Outlook

There have been three truly landmark events in the history of glutamate receptor research: the identification of the first selective NMDA receptor antagonists by Watkins and colleagues in the early 1980s, the cloning of the first cDNA encoding an AMPA receptor subunit in 1989 by Heinemann's group, and in late 1998 the identification of the first structure for a ligand-binding site by Gouaux's group. The first two discoveries triggered an avalanche of progress in the ensuing years; we can expect the same from the structural advance.

Much progress has been made in the past 5 years in the identification of the subunits and structural elements important for 1) transmembrane topology, 2) three of the four elemental functions of these receptors (i.e., ligand recognition, desensitization, and ion permeation), 3) receptor modulation by drugs, and 4) the functional consequences of cytoplasmic modifications. The fourth elementary function of ligand-gated ion channels, gating, has so far proved recalcitrant to study, although recent data suggest that new insights may be close at hand.

NMDA and AMPA receptors have received the most intense study, probably because their cDNAs were the first to be cloned. During the same time period the first wave of transgenic, knockout and knockin animals have

been generated, mostly for the NMDA receptors; results from these mice confirm the wide range of functional, behavioral, and developmental roles for glutamate receptors. The early clinical trials for treating stroke with NMDA or AMPA receptor antagonists have been a disappointment, in part due to the inability to administer a therapeutic dose of drug without adverse effects appearing. However, second-generation antagonists for AMPA and NMDA receptors are being introduced, leading to guarded optimism. Selective antagonists for kainate receptors are just now being developed. Clinical trials for relief of chronic pain by kainate and NMDA receptor antagonists are being planned, and currently it appears that pain may be the most likely initial indication to be successful clinically. For all of these reasons, the biology of glutamate receptors continues to attract significant attention and resources in biomedical research.

To date, all pharmacological approaches that manipulate glutamatergic synaptic transmission have involved the use of receptor antagonists or modulators of desensitization. Given that different subunit combinations confer specific functional features to glutamate receptors, an alternative strategy would involve the judicious genetic manipulation of subunit expression with the objective of more selectively influencing the properties of synaptic receptors. Indeed, given sufficient knowledge of the functional consequences of changing subunit stoichiometry, this genetic approach, while currently still unfeasible, may eventually offer an alternative and perhaps more systematic means to alter receptor function than the search for new antagonists.

As a final comment, the insertion of genetics into the glutamate receptor field is furnishing new technical approaches for manipulating receptor activation, and is providing the expected return on the investment by the research community of time, careers, and money. One expects efforts in this field to pay off in the next 5 years or so with the introduction of a new generation of pharmaceuticals directed to some of the most difficult clinical problems in neurology—chronic pain and stroke. Continued effort and collaboration among neurologists, physiologists, anatomists, and molecular biologists, in addition to a good deal of capital, will be necessary to realize this goal.

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The GluR2 hypothesis: Ca(++)-permeable AMPA receptors in delayed neurodegeneration.

Bennett MV, Pellegrini-Giampietro DE, Gorter JA, Aronica E, Connor JA, Zukin RS.

Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461, USA.

Increased glutamate-receptor-mediated Ca++ influx is considered an important factor underlying delayed neurodegeneration following ischemia or seizures. Until recently, the NMDA receptor was the only glutamate receptor known to be Ca(++)-permeable. It is now well established that glutamate receptors of the AMPA type, encoded by a gene family designated GluR1-GluR4, exist in both Ca(++)-permeable and Ca(++)-impermeable forms, depending on their subunit composition and degree of RNA editing. Recombinant channels assembled without GluR2 are permeable to Ca++; channels assembled with (edited) GluR2 are Ca(++)-impermeable. AMPA receptors in most adult neurons are hetero-oligomers containing GluR2 subunits, but some neurons have GluR2-less, Ca(++)-permeable receptors. The "GluR2 hypothesis" predicts that a relative reduction in the expression of GluR2 results in enhanced Ca++ influx through newly synthesized AMPA receptors, thereby increasing neurotoxicity of endogenous glutamate. Recent observations indicate reduction in GluR2 expression and predict formation of Ca(++)-permeable AMPA receptors following global ischemia and kainate-induced status epilepticus; these changes are likely to be a major factor contributing to the delayed neurodegeneration that follows these pathological events. The delayed neurodegeneration appears to be primarily apoptotic. Thus, there are at least three strategies for neuroprotection: block of formation of GluR2-less receptors, which may be possible at several levels; block of the GluR2-less receptors themselves; and block of the subsequent apoptosis.

Publication Types:
- Review
- Review, Tutorial

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ELSEVIER SCIENCE FULL-TEXT ARTICLE

Levodopa-induced motor complications are associated with alterations of glutamate receptors in Parkinson's disease.

Calon F, Rajput AH, Hornykiewicz O, Bedard PJ, Di Paolo T.

Molecular Endocrinology and Oncology Research Center, Laval University Medical Center, Quebec, Canada.

Glutamate receptors were studied in the brains of controls and Parkinson's disease (PD) patients, of which 10 of 14 developed motor complications (dyskinesias and/or wearing-off) following levodopa therapy. (125)I-RTI binding to the dopamine transporter and dopamine concentrations show comparable nigrostriatal denervation between the subgroups of PD patients. (3)H-Ro 25-6981 binding to the NR1/NR2B NMDA receptor was increased in the putamen of PD patients experiencing motor complications compared to those who did not (+53%) and compared to controls (+18%) whereas binding remained unchanged in the caudate nucleus. (3)H-AMPA binding was increased in the lateral putamen (+23%) of PD patients with motor complications compared to those without whereas it was decreased in the caudate nucleus of the PD patients (-16%) compared to controls. Caudate and putamen (3)H-CGP39653 binding to NR1/NR2A NMDA receptor and NR1 subunit mRNA levels measured by in situ hybridization were unchanged in subgroups of PD patients compared to controls. These findings suggest that glutamate receptor supersensitivity in the putamen plays a role in the development of motor complications (both wearing-Off and dyskinesias) following long-term levodopa therapy in PD.

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Centrally-administered AMPA antagonists increase locomotion in parkinsonian rats.

Stauch Slusher B, Rissolo KC, Anzilotti KF Jr, Jackson PF.

Department of Pharmacology, ZENECA Pharmaceuticals, Wilmington, DE, USA.

It was shown in the present study that three antagonists of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptor, including 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX), 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466) and 6-(1H-imidazole-1-yl)-7-nitro-2,3-(1H, 4H)-quinoxalinedione (YM90K), caused marked reversal of akinesia when administered into the entopeduncular nucleus of rats rendered parkinsonian by bilateral substantia nigra pars compacta lesion. These data suggest that centrally active AMPA antagonists may have therapeutic utility in the treatment of idiopathic Parkinson's disease.

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The link between excitotoxic oligodendroglial death and demyelinating diseases

Carlos Matute, Elena Alberdi, María Domercq, Fernando Pérez-Cerdá, Alberto Pérez-Samartín and María Victoria Sánchez-Gómez

Oligodendrocytes, the myelinating cells of CNS axons, are highly vulnerable to excitotoxic signals mediated by glutamate receptors of the AMPA and kainate classes. Receptors in these cells are commonly activated by glutamate that is released from axons and glial cells. In addition, oligodendrocytes contribute to the control of extracellular glutamate levels by means of their own transporters. However, acute and chronic alterations in glutamate homeostasis can result in overactivation of AMPA and kainate receptors and subsequent excitotoxic oligodendroglial death. Furthermore, demyelinating lesions caused by excitotoxins can be similar to those observed in multiple sclerosis. This, together with the effect of AMPA and kainate receptor antagonists in ameliorating the neurological score of animals with experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis), indicates that oligodendrocyte excitotoxicity could be involved in the pathogenesis of demyelinating disorders.

Carlos Matute*
Elena Alberdi
María Domercq
Fernando Pérez-Cerdá
Alberto Pérez-Samartín
María Victoria Sánchez-Gómez
Departamento de Neurociencias, Universidad del País Vasco, 48940-Leioa, Spain.
*e-mail: onpmaalc@lg.ehu.es

Enhanced glutamate signaling can lead to excitotoxicity, a phenomenon whereby overactivation of glutamate receptors (GluRs) triggers cell death. Excitotoxicity was first described in the late 1950s in retinal neurons[1]. Later, Olney and co-workers[2] found that this vulnerability is shared by all central neurons that contain GluRs. Thereafter, glutamate excitotoxicity has been implicated in acute injury to the CNS and in chronic neurodegenerative disorders[3–5].

The concept of excitotoxic cell death has recently been expanded to CNS glia. Glial cells include astrocytes, oligodendrocytes and microglia, which are distributed throughout the CNS. Classical studies have assigned to glia various roles that contribute to the support of neuronal function[6]. In addition, in the past few years it has been shown that astrocytes and oligodendroglia can actively participate in neurotransmission[7–9]. Strikingly, oligodendrocytes, which myelinate axons and constitute the vast majority of cells in the white matter, are highly vulnerable to overactivation of GluRs (Refs 10–12). This feature led to the proposal that oligodendroglial excitotoxicity might also be involved in the pathogenesis of demyelinating diseases[13–15], which are characterized by the destruction of myelin, oligodendrocyte cell death and inflammation[16,17]. This review discusses the current knowledge of the determinants of glutamate signaling in oligodendrocytes, the vulnerability of these cells to glutamate excitotoxicity and the evidence pointing to the relevance of this process in demyelinating disorders of the CNS.

All major types of CNS glial cells participate in glutamate signaling

Glutamate activates ionotropic and metabotropic receptors present in neurons and glial cells. Ionotropic GluRs can directly mediate excitotoxicity[18]. According to pharmacological, electrophysiological and molecular properties, ionotropic GluRs are classified as AMPA (subunits GluR1–4), kainate (subunits GluR5–7 and KA1–2) and NMDA (subunits NR1 and NR2A–D) receptors[18–20]. However, it should be noted that in spite of this nomenclature, kainate activates both AMPA and kainate receptors[21].

AMPA and kainate receptors are commonly present in astrocytes and oligodendrocytes[22–25] as well as in microglia[26,27]. By contrast, NMDA receptors are rare or absent in these cells[23]. Importantly, AMPA receptors in differentiated oligodendrocytes (Table 1), both *in vitro* and *in situ*, lack the GluR2 subunit, a feature that renders them permeable to Ca^{2+} (Ref. 31). In addition, the kainate receptor subunit GluR6, which is expressed in oligodendrocytes is edited to a

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Table 1. Ionotropic glutamate receptor subunits in oligodendrocytes[a]

	Oligodendrocyte precursors *in vitro*[b]	GalC +/MBP + cells *in vitro*[c]	White matter oligodendrocytes[d]	Spinal cord myelin[e]
$GluR_1$	–	–	–	–
$GluR_2$	+	–	–	–
$GluR_3$	+	+	+	–
$GluR_4$	+	+	+	+
$GluR_5$	–	–	nd	nd
$GluR_6$	+	+	+	nd
$GluR_7$	+	+	+	nd
KA_1	+	+	+	nd
KA_2	+	+	+	nd

[a]CNS neurons can express all AMPA, kainate and NMDA receptor subunits, however the presence of each subunit displays large regional variability[18,19]. By contrast, NMDA receptors in oligodendrocytes are rare or absent[13,23,25].
[b]Purified cultures of oligodendrocyte precursors derived from the rat cerebral cortex[28].
[c]Purified cultures of differentiated oligodendrocytes [galactocerebroside C-positive (GalC +) and myelin basic protein-positive (MBP +)] obtained from rat optic nerve[29].
[d]White matter tracts include corpus callosum, fornix, optic nerve and spinal cord[13,30]. nd, not determined.
[e]From Ref. 30. nd, not determined.

low extent[32,33], a molecular property that again results in native kainate receptors that are more permeable to Ca^{2+} (Ref. 31). Consequently, the higher permeability of GluRs to Ca^{2+} in oligodendroglia makes these cells more susceptible to excitotoxicity (Box 1).

GluR activation is terminated by transporters[34], which take up glutamate into surrounding cells. Astrocytes are the major contributors to glutamate uptake in synaptic regions during normal synaptic function[35]. However, glutamate transporters are also present in the white matter, in which glutamate can be released from axons following electrical activity[36] and from astrocytes by Ca^{2+}-dependent mechanisms[37]. Glutamate levels in white matter are, in turn, regulated by glutamate transporters that are present in astrocytes, oligodendrocytes[38,39] and axons[24,40].

Overactivation of glutamate receptors is toxic to oligodendrocytes

GluR-mediated toxicity has been observed in an oligodendroglial cell line[10] and in differentiated oligodendrocytes *in vitro*[11,12]. This toxicity is triggered by overactivation of AMPA and kainate receptors[11,12,29] and, similar to neurons, it is directly related to Ca^{2+} influx subsequent to receptor activation[11,12,29] (Box 1). The fact that these experiments were made in pure cultures of oligodendrocytes indicates that excitotoxicity is caused by direct activation of oligodendroglial receptors, although the absence of axons limits the

Box 1. Excitotoxicity and Ca^{2+} homeostasis

Cellular Ca^{2+} overload is a key trigger of neuronal death[a]. High concentrations of extracellular glutamate, generated after traumatic or ischemic CNS injury, results in overstimulation of ionotropic glutamate receptors (GluRs) and consequently, in an excessive influx of Na^+ and Ca^{2+}. Subsequently, the Na^+ influx can, in turn, trigger a secondary increase in the intracellular Ca^{2+} concentration through voltage-gated Ca^{2+} channels and reverse operation of the Na^+/Ca^{2+} exchanger[a,b]. In neuronal cultures, glutamate toxicity is mostly prevented by NMDA receptor antagonists[c]. However, AMPA/kainate receptors are also relevant to neuronal excitotoxicity[d]. Similarly, Ca^{2+} overload in glial cells, initiated by glutamate signals, can also trigger cell death[e].

The types of excitotoxic cell death observed depend on the intensity of the exposure and involve two temporally distinct phases of necrosis and apoptosis, a feature that relies on mitochondrial physiology[f]. The initial loss of mitochondrial membrane potential might be an important signal for apoptosis as long as ATP levels are sufficient to allow caspase 3 activation[g]. Mitochondria accumulate much of the Ca^{2+} that enters the cell during NMDA receptor activation. Ca^{2+} accumulation and oxidative stress cause mitochondrial depolarization and Ca^{2+} release back into the cytoplasm, a process that precedes neuronal death[h].

Ca^{2+} buffering by binding-proteins prevents excitotoxicity[i]. Interestingly, oligodendrocytes do not express several of the Ca^{2+}-binding proteins that are present in neurons[j]. This might explain why oligodendrocytes that express AMPA and kainate receptors, but not those of the NMDA receptor class, are equally vulnerable to excitotoxic insults compared with neurons.

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j Baimbridge, K.G. *et al.* (1992) Calcium-binding proteins in the nervous system. *Trends Neurosci.* 15, 303–308

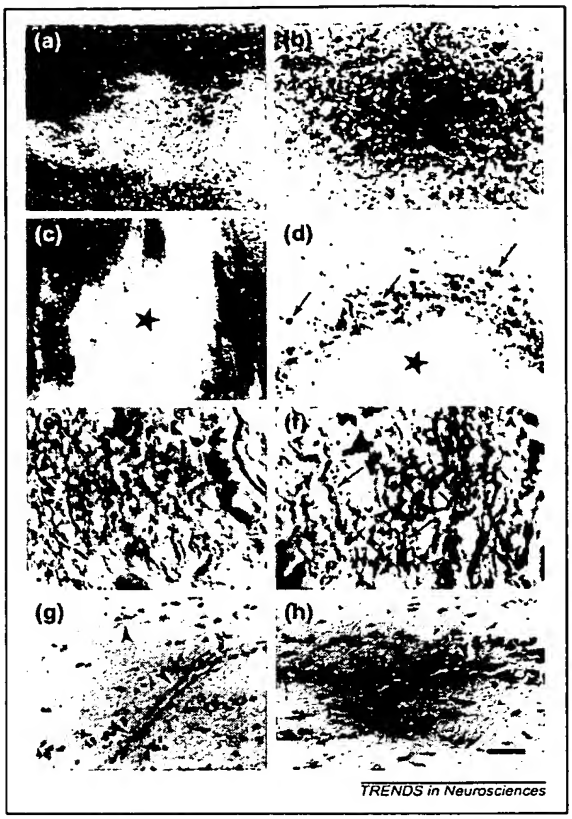


Fig. 1. Kainate-induced lesions in the optic nerve have the major features of multiple sclerosis. Slow prolonged application of moderate concentrations of kainate (100 μM) onto the optic nerve *in vivo* produces severe histological damage and demyelination in well circumscribed areas or plaques. These plaques have few oligodendrocytes as revealed with an antibody to 2′–3′ cyclic nucleotide 3′ phophodiesterase in (a) and (b) (CNPase, an oligodendrocyte marker; asterisks denote the same area at different magnification). Plaques (star) are demyelinated, as indicated by the lack of myelin basic protein (MBP) (c), and show numerous TUNEL [terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling] cells at the edge of the lesion (arrows) (d). Neurofilament immunostaining in treated nerves (f) reveals fiber alterations (arrows) and abundant axonal swellings (arrowheads) as observed in MS (Ref. 49), which are infrequent in control nerves (e). Nissl staining of vessels (arrowheads) shows an increased number of perivascular cells within a damaged area (h) compared to vessels in control nerves (g). Kainate was applied under the optic nerve duramater of young adult rabbits, from a cannula that was attached to an osmotic pump (delivery rate 0.5–1 µl/hr; 2–6 days). The nerves illustrated here were examined at 4–7 days after initiating the treatment with kainate or vehicle (control nerves). Scale bars, 140 µm in (a) and (b), 70 µm in (d), 14 µm in (e) and (f), and 35 µm in (b), (g) and (h). Modified, with permission, from Ref. 41.

physiological relevance of the results obtained in these studies. However, excitotoxic oligodendroglial death also occurs in isolated spinal dorsal columns[30], and *in vivo* following infusion with AMPA/kainate receptor agonists onto the optic nerve[11,41] and into the subcortical white matter[12].

An overwhelming amount of evidence indicates that neuronal excitotoxicity is the predominant mechanism underlying ischemic damage[5,42]. Similarly, immature and differentiated oligodendrocytes in culture are highly vulnerable to a transient deprivation of oxygen and glucose, an effect that is attenuated by AMPA and kainate antagonists[12,43,44]. The fact that these antagonists also protect against the ischemic damage that occurs in pure cultures of oligodendrocytes[44] indicates that these cells release glutamate by reversal of glutamate transport, a mechanism reported in hippocampal slices[45] and in spinal cord white matter[40] during ischemia and anoxia, respectively.

Consistent with these *in vitro* observations, double-labeling experiments *in vivo*, using *in situ* end-labeling of fragmented DNA and cell-type markers, show that ischemic insults can induce rapid oligodendroglial death[46]. A dramatic case of hypoxia-ischemia-related disease is cerebral palsy, in which lesions most frequently involve periventricular white matter[47]. Finally, glutamate spill-over from affected cells and axons in traumatic and anoxic lesions to the CNS can also significantly damage the oligodendroglial population[40,48].

Relevance of oligodendroglial excitotoxicity to demyelinating disorders of the CNS

The observations described above indicate that overactivation of AMPA and kainate receptors causes injury to oligodendrocytes both *in vitro* and *in vivo*, and that this toxicity is relevant to acute insults such as ischemia. Is this oligodendroglial death mechanism also involved in the pathogenesis of demyelinating diseases of the CNS? These disorders comprise those central white matter diseases with a suspected or confirmed autoimmune basis[16]. Among these, the most devastating is multiple sclerosis (MS), which is characterized by focal lesions with inflammation, demyelination and oligodendroglial death[16] resulting in inadequate nerve conduction and the appearance of neurological deficits.

Prolonged GluR overactivation leads to severe demyelination

GluR agonists, such as kainate, applied *in vivo* onto the optic nerve cause AMPA and kainate receptor-mediated histological damage[41], including disruption of the typical arrangement of interfascicular oligodendrocytes and gliosis. However, the type of lesion depends on the duration of the excitotoxic insult. Thus, a brief infusion of excitotoxin induces apoptotic oligodendroglial death and damage that does not result in long-term macroscopic alterations. By contrast, an infusion of kainate that lasts for several days causes extensive lesions with massive oligodendrocyte death, demyelination in plaques, axonal damage and inflammation (Fig. 1). Importantly, long-term effects of prolonged excitotoxic insults result in atrophy and profound demyelination. Such histopathological features are present in MS. Moreover, the different outcomes associated with short- and long-term consequences of excitotoxicity indicate that CNS axonal tracts appear to have repair mechanisms that are effective after

Table 2. Major features of multiple sclerosis, experimental autoimmune encephalomyelitis and excitotoxicity[a]

	MS and chronic EAE[b]	Acute demyelinating diseases and acute EAE[c]	Excitotoxicity[d]
Clinical course	Relapses and remissions	Monophasic	Progressive
Symptoms (motor and/or visual impairments)	Present	Present	Present[e]
Pathology			
Inflammation	Present	Present	Present
Demyelination	Present (MS >EAE)	Present (little in EAE)	Present
Oligodendrocyte loss	Present	Present	Present
Axonal damage	Present	Present	Present
White matter atrophy	Present	Absent	Present
T cells reactive to and/or antibodies to myelin	Present	Present (weak)	nd
Treatment with AMPA/kainate antagonists	nd	Improvement in EAE	Prevention

[a]Abbreviations. EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; nd, not determined.
[b]Chronic EAE is comparable to relapsing-remitting MS (Refs 16,52).
[c]Acute EAE is comparable to human acute demyelinating diseases, including acute optic neuritis, acute disseminated encephalomyelitis, and acute transverse myelitis. It is not clear if these diseases represent initial attacks of MS (Ref. 52) or are not related to acute MS (Ref. 16). Data from Refs 14–16,52.
[d]Data from Refs 13,30.
[e]Amplitude and profile of visual-evoked potentials in rabbits are reduced and distorted, respectively[54].

acute insults but are compromised in chronic injury. Indeed, focal demyelinating lesions in the adult brain can be remyelinated by endogenous oligodendrocyte progenitors, which differentiate into myelinating oligodendrocytes[50].

An important question arising from these results is whether excitotoxic damage is caused by direct excitotoxic oligodendroglial death or as a result of triggering an inflammatory response; the answer to this question is unknown at present. Given the fact that GluRs are present in oligodendrocytes, astrocytes and microglia, several simultaneous and different responses can occur within and among these cell populations following GluR overactivation. Among these responses, two might have special relevance to the damage observed. First, as described above, oligodendrocytes are directly vulnerable to excitotoxic insults. Second, GluR activation in microglia leads to the release of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) from these cells[27], which in turn can kill oligodendrocytes[51]. Accordingly, it is probable that following prolonged GluR overactivation, both direct oligodendroglial excitotoxicity and inflammation contribute to the damage observed in CNS axonal tracts. However, the fact that acute excitotoxic insults result in both rapid oligodendroglial death[12] and limited inflammation[41] indicates that the initial oligodendroglial excitotoxicity, as opposed to the oligodendroglial excitotoxicity secondary to astrocyte and microglial activation, might be the major trigger of demyelination found in chronic lesions.

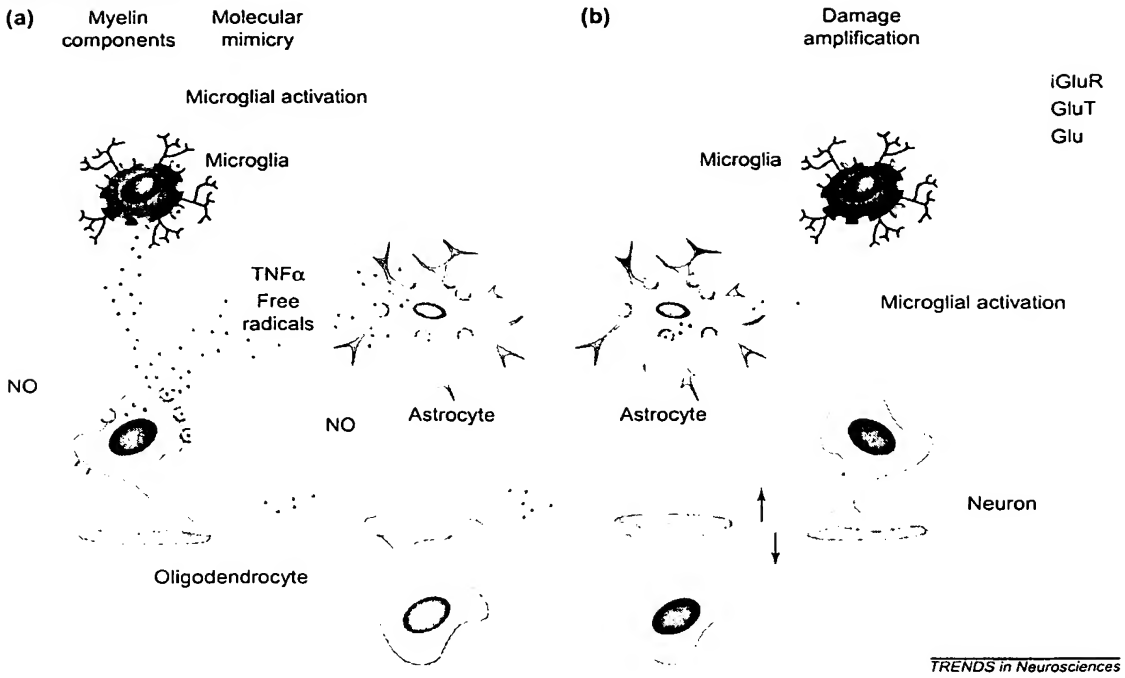
The presence of GluRs in the axoplasm as well as in oligodendrocytes and myelin[30], raises the question of whether axonal damage subsequent to excitotoxic insults occurs either directly or as a consequence of oligodendroglial death and demyelination. Although the former possibility can not be ruled out, it appears that oligodendrocytes die and myelin degenerates before the appearance of axonal damage upon exposure to GluR agonists, as observed in isolated spinal cord white matter[30]. In addition, the presence of GluRs in the myelin sheath[30] raises the possibility that this structure could be directly damaged by glutamate, independently of oligodendrocyte injury. This suggests that axonal damage is secondary to demyelination by excitotoxicity.

Together, these findings substantiate the hypothesis that an aberrant enhancement of glutamate signaling in white matter tracts represents a pathogenic component in chronic demyelinating diseases such as MS (Ref. 13).

Excitotoxic damage underlies part of the neurological deficits in experimental autoimmune encephalomyelitis

The idea proposed above is strongly supported by recent data obtained using experimental autoimmune encephalomyelitis (EAE). EAE, and in particular its chronic relapsing form, is perhaps the best characterized animal model of MS (Ref. 52). This disorder can be induced in genetically susceptible mammals, including non-human primates, by immunization with myelin proteins, or with T cell clones specific for certain peptides within these proteins[53]. This procedure alters the regulatory mechanisms that prevent autoimmunity and, similar to MS, this results in the infiltration of inflammatory cells to the CNS. Animals with chronic EAE exhibit many of the clinical and pathological features of MS (Table 2). This animal model has been useful in the development of new therapeutic agents to treat MS, including glatiramer acetate-copaxone® (Ref. 55), a synthetic polymer analog of myelin basic protein that has been approved for the treatment of MS.

The neurological symptoms in several forms of EAE are ameliorated by AMPA and kainate receptor antagonists[14,15]. In one of these studies[14], mice acquired EAE after transfer of lymph node cells that had been sensitized to myelin basic protein. The improvement in the clinical score observed in these animals following treatment with an AMPA/kainate receptor antagonist correlates with an increase in oligodendrocyte survival and reduced axonal damage. However, these beneficial effects are noted at an early stage of the disease, which does not usually involve demyelination. The other study[15] used the Lewis rat model of acute EAE, in which little demyelination occurs, together with a chronic EAE model. In both models, the secondary degeneration of spinal cord motor neurons, occurring as a consequence of the loss of white matter, was significantly reduced after treatment with GluR antagonists[15]. Unfortunately,



neuronal versus oligodendroglial death and axonal loss were not simultaneously examined and no evaluation of demyelination was carried out in these studies. Therefore, the putative contribution of each type of damage to the clinical outcome cannot be assessed. Nevertheless, these findings open up new avenues for the treatment of autoimmune demyelination.

Glutamate levels are elevated in MS

These remarkable findings raise several intriguing questions. Perhaps the most crucial of which is whether glutamate levels are increased in the CNS of patients with demyelinating disorders. Indeed, the concentration of this excitatory amino acid in cerebrospinal fluid (CSF) is higher in acute compared with silent MS and in controls[56], and it is associated with the severity and course of the disease[57].

A potential cellular source that can contribute to enhanced glutamate levels in CSF is activated microglia[58,59]. These cells, in cultures exposed to high K^+, can release glutamate via reverse glutamate transport, a process that it is potentiated under pathological conditions[59]. Oxidative stress might also contribute to the increase in glutamate concentration in the extracellular space, because free radicals reduce the efficiency of glutamate transporters[60]. Consistent with this possibility, the neurological deficit resulting from EAE has generally been reduced by trial therapies that are intended to diminish the concentration of reactive oxygen species[61].

Which comes first: excitotoxicity or autoimmunity?

Another question relates to the contribution of excitotoxicity to MS and possibly to other demyelinating disorders (Fig. 2). Is excitotoxicity just secondary to the autoimmune reaction, or alternatively could autoimmunity be triggered by focal excitotoxicity? Evidence in favor of the former possibility relies on the efficacy of AMPA/kainate antagonists in improving the neurological score in EAE. This indicates that a substantial part of the cellular damage is caused by excitotoxicity ensuing the immune reaction to myelin components[14,15]. The initial cell loss can be amplified by indirect oligodendroglial death caused by GluR-mediated release of TNFα from microglia[27] and the generation of reactive oxygen and nitrogen species by these cells[17,61] that, in turn, reduce glutamate uptake[60,62] (Fig. 2a). Thus excitotoxicity, secondary to autoimmunity, could indeed underlie a substantial part of the lesions observed in MS.

1: J Chem Neuroanat. 2002 May;23(4):249-53.

Related Articles, Links

ELSEVIER SCIENCE FULL-TEXT ARTICLE

Parvalbumin-containing interneurons in rat hippocampus have an AMPA receptor profile suggestive of vulnerability to excitotoxicity.

Moga D, Hof PR, Vissavajjhala P, Moran TM, Morrison JH.

Kastor Neurobiology of Aging Laboratories, Mount Sinai School of Medicine, Box 1639, One Gustave L. Levy Place, New York, NY 10029-6574, USA.

alpha-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors mediate excitatory neurotransmission in the central nervous system, and contain combinations of four subunits (GluR1-4). We developed a GluR3-specific monoclonal antibody and quantified the cellular distribution of GluR3 in rat hippocampus. GluR3 immunoreactivity was detected in all pyramidal neurons and most interneurons. In addition, we found a subset of parvalbumin (PV)-containing interneurons in the hippocampus and neocortex that was notable for its intense GluR3 immunoreactivity and lack of GluR2 immunoreactivity. Such an expression pattern of AMPA receptor subunits is likely to make these interneurons selectively vulnerable to excitotoxicity.

Publication Types:

- Review
- Review Literature

PMID: 12048108 [PubMed - indexed for MEDLINE]

TI: Survival of newly postmitotic motoneurons is transiently independent of exogenous trophic support.
AU: Mettling,-C; Gouin,-A; Robinson,-M; el-M'Hamdi,-H; Camu,-W; Bloch-Gallego,-E; Buisson,-B; Tanaka,-H; Davies,-A-M; Henderson,-C-E
SO: J-Neurosci. 1995 Apr; 15(4): 3128-37
IS: 0270-6474
LA: English
AB: We compared the survival requirements of early- and late-born motoneurons from E5 chicken spinal cord. Density gradient centrifugation followed by immunopanning using SC1 antibody allowed us to purify two size classes of motoneuron. Large motoneurons retained by 6.8% metrizamide were shown by BrdU labeling in ovo to be born on average 1.5 d earlier than the small motoneurons recovered from the metrizamide pellet. Large motoneurons were both biochemically and functionally more mature: they expressed higher levels of choline acetyltransferase and low-affinity neurotrophin receptor, and had an acute requirement for trophic support from muscle-derived factors. After 24 hr in culture in basal medium, all early-born motoneurons died, whereas 60% of late-born motoneurons survived. Small motoneurons can develop into large motoneurons in ovo, suggesting that they represent a general transitional stage in motoneuron development. Our results suggest that a defined period elapses between birth of a motoneuron and its acquisition of trophic dependence, possibly corresponding to the time required for target innervation. This property may have important consequences for the timing and regulation of developmental motoneuron death.
AN: 95239350

1: J Cereb Blood Flow Metab. 1988 Aug;8(4):474-85.

Related Articles, Links

Focal brain ischemia in the rat: methods for reproducible neocortical infarction using tandem occlusion of the distal middle cerebral and ipsilateral common carotid arteries.

Brint S, Jacewicz M, Kiessling M, Tanabe J, Pulsinelli W.

Department of Neurology, Cornell University Medical College, New York, NY 10021.

This article describes a 3-year experience with focal neocortical ischemia in three rat strains. Multiple groups of adult Wistar (n = 50), Fisher 344 (n = 31), and spontaneously hypertensive (n = 72) rats were subjected to permanent occlusion of the distal middle cerebral (MCA) and ipsilateral common carotid arteries (CCA). Twenty-four hours later the animals were killed, and frozen brain sections were stained with hematoxylin and eosin to demarcate infarcted tissue. The infarct volume for each section was quantified with an image analyzer, and the total infarct volume was calculated with an iterative program that summed all interval volumes. Neocortical infarct volume was the largest and most reproducible in the spontaneously hypertensive rats (SHR). Statistical power analysis to project the numbers of animals necessary to detect a 25 or 50% change in infarct volume with alpha = 0.05 and beta = 0.2 revealed that only the SHR model was practical in terms of requisite animals: i.e., less than 10 animals per group. Tandem occlusion of the distal MCA and ipsilateral CCA in the SHR strain provides a surgically simple method for causing large neocortical infarcts with reproducible topography and volume. The interanimal variability in infarct volume that occurs even in the SHR strain dictates that randomized, concomitant controls are necessary in each study to ensure the accurate assessment of experimental manipulations or pharmacologic therapies.

PMID: 3392112 [PubMed - indexed for MEDLINE]

22096
Ser. No.
10/030,436

TI: Survival of newly postmitotic motoneurons is transiently independent of exogenous trophic support.
AU: Mettling,-C; Gouin,-A; Robinson,-M; el-M'Hamdi,-H; Camu,-W; Bloch-Gallego,-E; Buisson,-B; Tanaka,-H; Davies,-A-M; Henderson,-C-E
SO: J-Neurosci. 1995 Apr; 15(4): 3128-37
IS: 0270-6474
LA: English
AB: We compared the survival requirements of early- and late-born motoneurons from E5 chicken spinal cord. Density gradient centrifugation followed by immunopanning using SC1 antibody allowed us to purify two size classes of motoneuron. Large motoneurons retained by 6.8% metrizamide were shown by BrdU labeling in ovo to be born on average 1.5 d earlier than the small motoneurons recovered from the metrizamide pellet. Large motoneurons were both biochemically and functionally more mature: they expressed higher levels of choline acetyltransferase and low-affinity neurotrophin receptor, and had an acute requirement for trophic support from muscle-derived factors. After 24 hr in culture in basal medium, all early-born motoneurons died, whereas 60% of late-born motoneurons survived. Small motoneurons can develop into large motoneurons in ovo, suggesting that they represent a general transitional stage in motoneuron development. Our results suggest that a defined period elapses between birth of a motoneuron and its acquisition of trophic dependence, possibly corresponding to the time required for target innervation. This property may have important consequences for the timing and regulation of developmental motoneuron death.
AN: 95239350

TI: Cardiotrophin-1 requires LIFRbeta to promote survival of mouse motoneurons purified by a novel technique.
AU: Arce,-V; Garces,-A; de-Bovis,-B; Filippi,-P; Henderson,-C; Pettmann,-B; deLapeyriere,-O
SO: J-Neurosci-Res. 1999 Jan 1; 55(1): 119-26
IS: 0360-4012
LA: English
AB: The cytokines ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) signal through a receptor complex formed between two transmembrane proteins, gp130 and LIFRbeta. In addition, CNTF also uses a ligand-binding component which is anchored to the cell membrane. In the case of cardiotrophin-1 (CT-1), LIFRbeta is also required in cardiomyocytes, but this has not been proven in neurons, and published data suggest that motoneurons may use a different receptor complex. We used Lifrbeta knockout mice to assess the requirement for this receptor component in the signal transduction of CT-1 in motoneurons. To study purified motoneurons from such mutants, we have developed a method allowing for isolation of highly purified mouse motoneurons. This protocol is based on the immunoaffinity purification of motoneurons using antibodies against the extracellular domain of the neurotrophin receptor, p75, followed by cell sorting using magnetic microbeads. We show that CNTF, LIF, and CT-1 are unable to promote the survival of motoneurons derived from homozygous Lifrbeta-/- mutant embryos. Thus, LIFRbeta is absolutely required to transduce the CT-1 survival signal in motoneurons.
AN: 99105446

22096
Ser. No.
10/030,436

TI: The neuroprotective activity of 8-alkylamino-1,4-benzoxazine antioxidants.
AU: Largeron,-M; Mesples,-B; Gressens,-P; Cecchelli,-R; Spedding,-M; Le-Ridant,-A; Fleury,-M
SO: Eur-J-Pharmacol. 2001 Jul 27; 424(3): 189-94
IS: 0014-2999
LA: English
AB: Antioxidant 8-alkylamino-1,4-benzoxazines, (R,S)-(3-tert-butyl-8-phenylethylamino-3,4-dihydro-2H-1,4-benzoxazin-5-yl) (phenyl) methanone (S 24429) and (R,S)-(3-cyclopentyl-8-benzylamino-3,4-dihydro-2H-1,4-benzoxazin-5-yl) (phenyl) methanone (S 24718), were prepared according to a two-step one-pot electrochemical procedure. These compounds had been selected from a previous study of structure/activity. Both compounds (1-100 microM) prevented the fall in ATP levels caused by 24 h of hypoxia in astrocytes. Both compounds (1 and 10 mg/kg i.p.) were powerful neuroprotective agents in protecting against the lesions induced by 15 microg S-bromo-willardiine injected into the cortex or white matter of 5-day old mice pups. In contrast, exifone, an antioxidant compound, was inactive at these doses. S 24429 and S 24718 appear to be novel neuroprotective agents, which are effective in a model of brain damage mimicking the lesions underlying cerebral palsy.
AN: 21526859

Phenotyping of Drug Metabolism in Infants and Children: Potentials and Problems

Anders Rane, MD, PhD

ABBREVIATION. PM, poor metabolizer.

Genetic polymorphism in drug-metabolizing enzymes is a predominant cause of variability in drug metabolism, along with physiologic, pathophysiologic, and environmental factors (Table 1). Such polymorphisms are of interest from a basic biomedical, as well as a clinical, point of view, because differences in treatment outcome and adverse drug reactions have been associated with the different phenotypes.

Increased knowledge in this field should also be of great interest in pediatric drug therapy. However, there is almost no information about the maturation of polymorphic traits during ontogenesis. This issue has therapeutic implications in pediatrics, first, because several drug substrates of the polymorphic enzymes also are used in infants and children, and second, because for many such drugs, the treatment results may not be monitored by objective parameters.

A number of polymorphisms in drug metabolizing enzymes have been discovered in the last few decades (Table 2). The cytochrome P450 (CYP) family is the major enzyme system for oxidation of drugs. The clinically most important polymorphisms in this family include the debrisoquine/sparteine type in the CYP2D6 enzyme and the mephenytoin type in the CYP2C19 enzyme. They are based on detrimental mutations in the enzyme genes. Pharmacogenetic polymorphisms also have been described in phase II enzymes, eg, *N*-acetyl transferase, which was among the first to be discovered. Other polymorphisms have been described for members of the glutathione *S*-transferase enzyme family, but the clinical importance of these for drug therapy is not fully understood.

THE CYP2D6 (DEBRISOQUINE/SPARTEINE) POLYMORPHISM

This polymorphism is inherited as an autosomal recessive trait.[1] Homozygous mutated individuals are denoted as poor metabolizers (PM) and are deficient in their metabolism of a variety of drugs (Table 3). The CYP2D6 polymorphic metabolism pattern involved many important groups of drugs, eg, several β-adrenoceptor-blocking agents,[2–4] antidepressants,[5] and opiates.[6–8]

At present, little is known about the development of the extensive metabolizer phenotype during ontogenesis. In vitro studies in human fetal liver microsomes in our laboratory have revealed that the *N*-demethylation of codeine and dextromethorphan precedes the development of the *O*-demethylation reaction.[9] Whereas the *N*-demethylation is catalyzed by the nonpolymorphic CYP3A, the *O*-demethylation of these drugs is catalyzed by CYP2D6. Studies by Trelyuer et al[10] indicate that the CYP2D6 enzyme is expressed in a minority of liver specimens from late gestational period or from newborn infants. The gene expression of this enzyme seems to precede the formation of the enzyme protein. Only in adulthood is there a positive correlation between mRNA and enzyme protein, suggesting a transcriptional regulation in adults. Trelyuer et al[10] found that the catalytic in vitro activity of CYP2D6 developed postnatally over a period of several months.

THE CYTOCHROME P4502C19 (MEPHENYTOIN-TYPE) POLYMORPHISM

The polymorphism was discovered in studies of the kinetics of the antiepileptic drug mephenytoin.[11] The CYP2C19 enzyme catalyzes the oxidation of many psychoactive drugs, antiepileptics, and so forth (Table 4). It is deficient in 2% to 5% of whites.[12] Studies in different populations have revealed ethnic differences in the prevalence of the deficient. PM phenotype. Approximately 15% to 20% of Chinese and Japanese individuals are PMs.[13,12] The enzyme deficiency is inherited as an autosomal recessive trait. No studies on this polymorphism during human development have been found.

THE *N*-ACETYL TRANSFERASE POLYMORPHISM

Adult subjects are grouped into slow or rapid acetylators according to their capacity to acetylate isoniazid, sulfonamides, dapsone, or other probe drugs of this enzyme. The frequency of slow acetylators varies widely among different ethnic groups, from 50% to 70% in whites[14,15] to <25% in the Japanese population.[14]

From the Department of Clinical Pharmacology, University Hospital, Uppsala, Sweden.
Received for publication Mar 30, 1999; accepted Mar 31, 1999.
Address correspondence to Anders Rane, MD, PhD, Department of Clinical Pharmacology, University Hospital, S-751 85 Uppsala, Sweden.
PEDIATRICS (ISSN 0031 4005).

TABLE 1. Causes of Variation in Drug Elimination

Variation in renal excretion (small at normal kidney function)
Variation in liver metabolism (significant, even at normal liver function)
Environmental factors
diet
pollutants
alcohol, tobacco, etc
drugs
Age
Heredity (monomorphic and/or polymorphic variation)

Diverging data on the maturation of the rapid acetylation trait have been published. The rapid acetylation phenotype is expressed when none or only one allele of the NAT2 gene carries a mutation.[15] We investigated the in vitro acetylation of 7-amino-clonazepam in human fetal and adult liver preparations.[16] Whereas the adult enzyme preparations were possible to classify in slow and rapid acetylators, no such dichotomy was observed in the fetal enzyme preparations.

However, the fetal enzyme may be very active toward certain substrates. Meisel et al[17] found that the fetal liver enzyme activity (gestational week 9 to 12) was close to 50% of the adult fast acetylation rate using procainamide as substrate. We have no explanation for the apparent discrepancy between the results other than the possibility that different enzymes may be involved in the two reactions.

Several confounding factors are possible in experiments with human fetal tissue specimens. The postmortem enzyme degradation may vary among liver specimens. The effect of gestational age also may conceal a possible in vitro polymorphism. And finally, the endocrine influence of the pregnant woman may contribute to the suppression of a polymorphic enzyme expression, as may postnatal conformational changes in the enzyme protein, as suggested by Cohen and Weber.[18]

Our in vitro results are consistent with attempts to phenotype infants and children with caffeine as probe drug of the *N*-acetyl transferase 2.[19] In a group of 14 infants, it appeared that all but one (the oldest) were slow acetylators, using an antimode of 0.4. One infant was identified as a slow acetylator at age 54 days, but turned into a rapid acetylator phenotype at age 7 months. Carrier et al[19] concluded that the *N*-acetyl transferase is immature and that caffeine acetylation phenotype cannot be determined with certainty in infants younger than age 1 year.

The maturation of caffeine acetylation was studied subsequently by Pariente-Kayat et al[20] using caffeine as probe drug. They included 54 children, 8 to 447 days of age, who were admitted to hospital for minor diseases. A group of 5 children with Pierre Robin syndrome also was included. The cumulative percentage of rapid acetylators increased as a function of age. The plateau still was not achieved at 15 months of age, as assessed by the AFMU/1X and AFMU/AFMU +1U, +1X, +7U, +1.7X ratios. It was concluded that acetylation status cannot be determined with certainty before age 15 months.

Sulfadimidine also has been used as probe drug in phenotyping acetylation rate.[21] With this drug, a significantly higher proportion (83%) of newborn infants belonged to the slow acetylator phenotype compared with adults (50%). Szorady et al[21] partly ascribed this difference to deficient dietary intake of pantotenic acid, which is required for coenzyme A in the reaction. Obviously, other reasons also may contribute to the age-dependent difference.

It is concluded that the slow phenotype predominates in newborn infants and infants during the first year. Slow postnatal maturation of the rapid acetylation phenotype may result in higher sensitivity of such infants to pharmacologic and toxic effects of drugs that are substrates of the *N*-acetyl transferase (Table 5). Uncritical use of these drugs may be a potential risk.

CONCLUSIONS AND PERSPECTIVE

As in adults, the variation in drug metabolism in infants and children is based on constitutional, genetic, and environmental factors. The existence of drug-metabolic polymorphisms gives an additional dimension to the variation, which is clinically important in the treatment with a variety of widely used drugs. Inasmuch as many of these drugs are used in infants and children, the phenotypic expression should be of even greater interest in these groups because effects and side effects of drugs in children are often not possible to monitor by objective means. The limited information about the functional maturation of the polymorphic enzymes should fuel increasing interest in this field. More information is needed to minimize the risk of therapeutic hazards in this age group. There are several apparent indications for phenotyping in clinical drug therapy (Table 6). If an enzyme path-

TABLE 2. Some Polymorphisms in Drug-metabolizing Enzymes

Enzyme	Function	Discovered Through	Genophenotyping*	Ref. No.
Pseudocholinesterase	Hydrolysis	Pronounced clinical effect (apnea) of sucinylcholin/suxamethonium	Kalow and Genest, 1957	22
N-acetyl-transferase	Acetylation	High concentration of isoniazid	Price Evans et al 1960	23
Cytochrome P450D6	Oxidation	Ortostatic hypotension of debrisoquine	Maghoub et al 1975	1
Cytochrome P4502C19	Oxidation	Pronounced sensitivity to mephenytoin	Küpfer and Preisig 1984	11
GSTM1	Conjugation	—	Seidegard et al 1986	24
GSTT1	Conjugation	—	Pemble et al 1994	25
GSTP1	Conjugation	—	Satoh et al 1985	26

TABLE 3. Drug Substrates of the Polymorphic Cytochrome P450 (CYP) 2D6 Enzyme

Cardiovascular agents	β-adrenoceptor blockers
Debrisoquine	Metoprolol
Sparteine	Propranolol
Propafenone	Timolol
Flecainide	Antiemetics
Mexiletine	Tropisetron
Neuroleptics	Tricyclic antidepressants
Haloperidol	Amitriptyline
Perphenazine	Nortriptyline
Thioridazine	Clomipramine/
Chlorpormazine	desmethylclomipramine
Remoxipride	impramine/
Zuklopenitoxol	Despiramine
Opiates	Other anatidepressants
Codeine	Paroxetine
Dextromethorphan	Fluxetine
Ethylmorphine	Desmethylcitalopram
	Mianserin

TABLE 4. Drug Substrates of the Polymorphic Cytochrome P450 (CYP) Enzyme

Antiepileptics	Antidepressants
Mephenytoin	Citalopram
Benzodiazepines	Imipramine
Diazepam/desmethyidizepam	Clomipramine
β-Adrenoceptor blockers	Moclobemide
Propranolol	Antimalarials
Antiulcer agents	Proguanil*
Omeprazole	

* Bioactivates by CYP2C19 to cycloguanil.

TABLE 5. Drug Substrates of the Polymorphic *N*-acetyltransferase (NAT2) Enzyme

Cardiovascular agents	MAO inhibitors
Procainamide	Phenelzine
Hydralazine	Tuberculostatics
Sulfonamides	Isoniazid
Dapsone	*P*-aminosalicylic acid
Sulfasalazine	Benzodiazepines
Sulfamethoxazole	Nitrazepam*
Sulfadiazine	Other agents
Sulfacetamide	p-Aminobenzoic acid
Antihormones	Caffeine*
Aminoglutethimide	

* Bioconverted to amines before acetylation.

TABLE 6. Indications for Phenotyping In

Adults	Children
Polymorphic pathway	Polymorphic pathway
Long-term treatment	Long-term treatment
Low therapeutic index	Low therapeutic index
Toxicity problems	Toxicity problems
Unexpected outcome of therapy	Unexpected outcome of therapy
Low or high concentration per dose unit	Low or high concentration per dose unit
Drug–drug interactions	Drug–drug interactions
	In Addition
	Page-dependent kinetics
	Signs of toxicity subjective
	Effect parameters not objective

way is polymorphic and the drug to be used is for long-term treatment, or if signs of toxicity are primarily subjective, or if effect parameters are not measurable by objective methods, phenotyping may help to individualize the dose according to the patient's need. Low therapeutic index, toxicity problems, unexpected outcome of therapy, and so forth, should also increase the motivation for phenotyping. Additional indications to phenotype infants and children are age-dependent kinetics of drugs that are polymorphically metabolized.

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AMPA receptor blockade improves levodopa-induced dyskinesia in MPTP monkeys

S. Konitsiotis, MD; P.J. Blanchet, MD, PhD; L. Verhagen, MD; E. Lamers, MD; and T.N. Chase, MD

Article abstract—*Objective:* To evaluate the contribution of amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) glutamate receptors to the pathogenesis of parkinsonian signs and levodopa-induced dyskinesias. *Background:* Motor fluctuations and dyskinesias reflect, in part, altered function of glutamate receptors of the NMDA subtype. The possible role of AMPA receptors, however, has not yet been examined. *Methods:* The authors compared the ability of an AMPA agonist (CX516) and a noncompetitive AMPA antagonist (LY300164) to alter parkinsonian symptoms and levodopa-induced dyskinesia in MPTP-lesioned monkeys. Eight levodopa-treated parkinsonian monkeys received rising doses of each drug, first in monotherapy and then in combination with low-, medium-, and high-dose levodopa. *Results:* CX516 alone, as well as when combined with low-dose levodopa, did not affect motor activity but induced dyskinesia. Moreover, following injection of the higher doses of levodopa, it increased levodopa-induced dyskinesia by up to 52% ($p < 0.05$). LY300164 potentiated the motor activating effects of low-dose levodopa, increasing motor activity by as much as 86% ($p < 0.05$), and that of medium-dose levodopa as much as 54% ($p < 0.05$). At the same time, LY300164 decreased levodopa-induced dyskinesia by up to 40% ($p < 0.05$). *Conclusions:* AMPA receptor upregulation may contribute to the expression of levodopa-induced dyskinesia. Conceivably, noncompetitive AMPA receptor antagonists could be useful, alone or in combination with NMDA antagonists, in the treatment of PD, by enhancing the antiparkinsonian effects of levodopa without increasing and possibly even decreasing levodopa-induced dyskinesia. **Key words:** PD—Dyskinesia—Levodopa—MPTP—Basal ganglia—Nonhuman primates—Animal models.

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The treatment of PD aims to restore striatal dopamine (DA)-mediated transmission with either the DA precursor levodopa or a direct DA receptor agonist.[1] This therapeutic strategy initially brings substantial symptomatic relief,[2] but with disease progression, treatment-related complications become increasingly prominent.[3]

Increasing evidence suggests that DA denervation-associated parkinsonian signs and treatment-associated motor complications may reflect, at least in part, alterations in the function of glutamate receptors expressed on striatal medium spiny neurons.[4,5] The distal dendrites of spiny neurons, which account for nearly all striatal neurons, receive cortical glutamatergic projections as well as nigral dopaminergic terminals.[6] In parkinsonian rats, the chronic nonphysiologic stimulation of striatal dopaminergic receptors has been observed to activate signaling pathways within spiny neurons that regulate the phosphorylation of coexpressed glutamatergic receptors, especially those of the NMDA subtype.[7,8] As a result, synaptic efficacy appears to rise and striatal output changes in ways that compromise motor function.[9] In support of these possibilities, pharmacologic observations in parkinsonian monkeys and PD patients indicate that drugs able to block NMDA receptors selectively can alleviate parkinsonian signs as well as palliate dopaminomimetic-induced motor complications.[10-15]

In addition to NMDA receptors, medium spiny neurons also express glutamatergic receptors of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) type.[16,17] Despite their dense expression at the dendritic spine heads,[18] as well as elsewhere in the basal ganglia,[19] these ionotropic receptors have received relatively scant investigative attention in relation to the regulation of motor function. Whereas AMPA receptor blockade with the competitive antagonist NBQX has no consistent effect on motor function when given alone to parkinsonian rats or monkeys,[20,21] some potentiation of the antiparkinsonian action of levodopa and apomorphine has generally been reported.[22,23] More recently, a competitive nonselective AMPA/kainate antagonist has also been observed to reduce the response alterations produced in rats by intermittent levodopa therapy.[24]

To evaluate further the contribution of AMPA receptor-mediated mechanisms to motor behavior in parkinsonian primates, we compared the effects of a selective agonist with those of a selective antagonist:

From the Experimental Therapeutics Branch, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD.
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Address correspondence and reprint requests to Dr. T.N. Chase, Experimental Therapeutics Branch, Building 10, Room 5C103, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD 20892-1406; e-mail: chase@helix.nih.gov

CX516, a benzoylpiperidine analog in a novel class of compounds commonly referred to as "ampakines," is able to enhance glutamatergic transmission by acting at an allosteric site to positively modulate AMPA receptors[25]; LY300164 is a noncompetitive antagonist that acts at an allosteric modulation site to block AMPA receptors.[26,27] Both systemically administered drugs were studied alone and in combination with levodopa in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned cynomolgus monkeys that manifested levodopa-induced dyskinesias.

Methods. *Animals.* Eight adult cynomolgus (*Macaca fascicularis*) monkeys of either sex, weighing 3.4 to 7.3 kg, were studied in accordance with a protocol approved by the NIH Animal Care and Use Committee. Animals were housed individually under stable room conditions with a 12-hour light/dark cycle. All were fed a standard biscuit diet twice daily supplemented with fruit and had free access to water. Each was injected subcutaneously with MPTP HCl (Research Biochemicals, Natick, MA) once a week at a dose of 0.5 to 1 mg/kg until definite parkinsonian features, including sustained tremor, appeared. The average cumulative MPTP dose was 4.4 mg/kg (range 2.1 to 9.75 mg/kg).

Animals were regularly scored on the Laval University Disability Scale for MPTP Monkeys,[28] where the normal state extends from 0 to 2 points and maximum disability is 10 points (see below). Once mild to moderate parkinsonian signs became stably present, MPTP injections were discontinued and the animals were observed for 2 additional months before initiation of oral levodopa/carbidopa treatment (Sinemet, 100 mg/25 mg tablets; Roane-Barker, Greenville, SC) given as a single daily dose of 0.5 to 2 tablets hidden in food pellets or fruit bites and ingested spontaneously. Within 4 to 5 weeks on this regimen, all monkeys began to manifest dyskinesias, which were subsequently reproduced with each oral dose of levodopa/carbidopa. A thrice-weekly maintenance dose of oral levodopa/carbidopa was then begun to sustain dyskinesias and avoid the confounding effect of levodopa withdrawal during subsequent studies. All animals studied had remained clinically stable under baseline (untreated) conditions for at least the preceding 2 years (Disability Scale score 4.5 ± 0.2 points; $p < 0.0001$ compared with pre-MPTP score). On testing days, the morning meal and oral levodopa/carbidopa dose were withheld.

Drug treatments. A dose-finding study of levodopa was conducted first. Rising daily doses of levodopa methyl ester (SC, increments of 25 to 50%) combined with benserazide (12 mg/kg SC; Research Biochemicals) were administered to determine for each animal a "low" dose just below the threshold for a clinical response (mean total dose 37 mg, range 10 to 150 mg), a "medium" dose that reversed all parkinsonian signs but produced only moderate dyskinesias (mean 109 mg, range 40 to 300), and a "high" dose sufficient to produce a greater dyskinetic response (mean 233 mg, range 60 to 500).

Dose finding studies were then performed with LY300164 (Eli Lilly, Indianapolis, IN) and CX516 (Cortex Pharmaceuticals, Irvine, CA) given alone to determine the maximum tolerated dose, a dose tenfold lower, and a dose midway between these two. When coadministered with levodopa, these doses of LY300164 (1, 5, and 10 mg/kg, PO) and CX516 (5, 50, and 100 mg/kg, SC) were always given 40 minutes before any of the predetermined doses of levodopa/benserazide. At the doses selected, no drugs or drug combinations produced clinically evident adverse effects in any animal.

An interval of at least 3 days separated each administration of a particular glutamatergic drug and a washout of 6 weeks separated different drug regimens. During the washout period, the three SC doses of levodopa were administered again to assure that the individual animal's response had not changed.

Levodopa/benserazide and CX516 were dissolved in sterile normal saline (containing ascorbic acid 0.2 mg/mL) for the levodopa solution, or 10% hydroxypropyl-β-cyclodextrin (Aldrich; Milwaukee, WI) for the CX516 solution, just before SC administration in the abdomen or flank, with the injection sites carefully rotated to avoid local irritation. LY300164 was loaded into gelatin capsules, placed in the animal's oropharynx with a pet piller, and swallowed spontaneously.

Response assessments. Both qualitative and quantitative response assessments to each drug were obtained. Monkeys were transferred to an observation room and allowed to habituate to their new environment as determined by stable rating scores on the Disability Scale. Following drug administration, behavioral ratings were performed in a blinded fashion by the same investigator every 15 minutes for 4 hours or until there was a complete return to baseline scores.

Parkinsonian severity was scored on the Disability Scale, which uses the following motor and behavioral items[28]: posture—normal = 0, flexed = 1, crouched = 2; mobility—normal = 0, passive = 1; climbing—present = 0, absent = 1; gait—normal = 0, abnormal = 1; tremor—absent = 0, present = 1; holding food—present = 0, absent = 1; vocalizing—present = 0, absent = 1; grooming—present = 0, absent = 1; social interaction—present = 0, absent = 1. Occasionally, a score of 0.5 was given to reflect very mild motor abnormalities or bodily asymmetry. A definite antiparkinsonian response was considered present as long as the baseline motor subscore (first five items) improved by at least two points. The magnitude of the antiparkinsonian response is reported as the sum of changes in Disability Scale scores during the 4 hours following drug administration. Animals were also observed for drug-related adverse events including emesis, somnolence, ataxia, or hallucinatory-like behavior.

Dyskinesias were scored using the Abnormal Involuntary Movements Scale[14,28]: occasional, mild = 1; intermittent, moderate = 2; continuous, severe = 3; intermediate scores (0.5, 1.5, 2.5) were allowed to reflect slight bodily asymmetries, and each segment (face, neck, trunk, each limb) was scored separately. Results are reported as a Dyskinesia Severity Index (DSI) calculated using the formula:

(sum of all dyskinesia scores/duration of antiparkinsonian effect) × 100[14,28]

When no antiparkinsonian response was observed, the duration of the dyskinetic response was used instead of the duration of the antiparkinsonian response as the denominator in the DSI formula.

Portable activity monitors (PAM; IMSystems, Baltimore, MD) tied underneath a primate jacket provided a continuous objective assessment of total motor activity re-

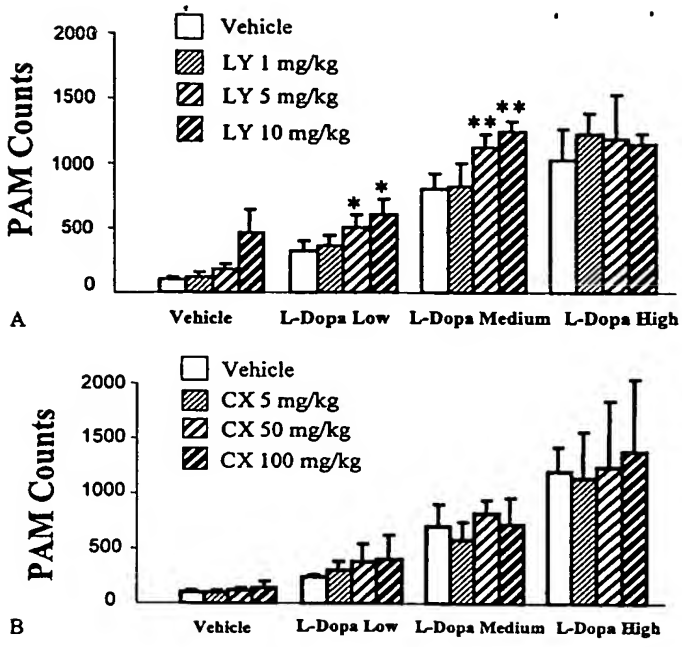

*Figure 1. Portable activity monitor (PAM) counts recorded over a period of 4 hours after treatment with levodopa (low-, medium-, or high-dose L-DOPA) alone, or in combination with (A) LY300164 (LY) or (B) CX516 (CX). Values are mean ± SEM. *p < 0.05 versus L-DOPA low alone, **p < 0.05 versus L-dopa medium alone.*

sulting from each drug administration. Each animal always used the same monitor. PAM counts were accrued for 4 hours following drug administration to measure total motor activity and are expressed as a percent of each animal's baseline value.

Statistics. The magnitude and duration of the response as determined by Disability Scale scores, total motor activity as determined by PAM counts accrued over a period of 4 hours, and the calculated DSI values were pooled for all animals and expressed as means ± SEM. Differences were determined using an ANOVA for repeated measures (the non-parametric Friedman's test being used for the DSI scores and the magnitude of the response) followed by a post hoc Dunnet's test. A p value < 0.05 was considered significant.

Results. *Antiparkinsonian response.* LY300164 monotherapy at all three tested doses (1, 5, and 10 mg/kg PO) had no significant antiparkinsonian effect, although at the highest dose (10 mg/kg) three of eight monkeys evidenced modest improvement in Disability Scale scores (ranging from 0.5 to 3.5 points) and for the entire group mean PAM counts tended to increase (367 ± 170; $p < 0.08$; figure 1A). CX516 (5, 50, and 100 mg/kg) alone failed to alter Disability Scale scores in any of the parkinsonian animals (figure 1B).

Coadministration of the higher doses of LY300164 potentiated the motor response to low-dose levodopa. Disability Scale ratings appeared to improve slightly at 5 mg/kg ($p < 0.08$; table 1) and PAM activity increased by 55% at 5 mg/kg and 86% at 10 mg/kg (both $p < 0.05$; see figure 1A). None of the doses of CX516 (5, 50, and 100 mg/kg) had any effect on the response to the low dose of levodopa (table 2, figure 1B). Combined with medium-dose levodopa, LY300164 again was found to potentiate antihypokinetic activity: PAM counts increased in a dose dependent manner by 38% at 5 mg/kg and 54% at 10 mg/kg (both $p < 0.05$; see figure 1A), although no consistent effect on Disability Scale scores could be documented (see table 1). Coadministration of medium-dose levodopa with CX516 did not affect the former drug's response profile (table 2, figure 1B). Neither LY300164 nor CX516 altered the response to the high dose of levodopa.

***Table 1** Magnitude and duration of the antiparkinsonian response (means ± SEM) in eight levodopa-primed parkinsonian monkeys after treatment with levodopa (low-, medium-, or high-dose L-DOPA) alone or in combination with LY300164 (LY)*

Drug A, mg/kg	Drug B	Response duration†	Response magnitude‡
Vehicle[a]	Vehicle[b]	0	0
Vehicle[a]	L-DOPA low	0	1 ± 1*
Vehicle[a]	L-DOPA medium	103 ± 3*	19 ± 1*
Vehicle[a]	L-DOPA high	151 ± 9*	22 ± 2*
LY (1)	Vehicle[b]	0	0
LY (5)	Vehicle[b]	0	0
LY (10)	Vehicle[b]	0	1 ± 1
LY (1)	L-DOPA low	10 ± 10	2 ± 1
LY (5)	L-DOPA low	25 ± 14	7 ± 3
LY (10)	L-DOPA low	13 ± 13	3 ± 1
LY (1)	L-DOPA medium	109 ± 21	17 ± 3
LY (5)	L-DOPA medium	143 ± 7	24 ± 2
LY (10)	L-DOPA medium	111 ± 21	19 ± 4
LY (1)	L-DOPA high	163 ± 6	26 ± 3
LY (5)	L-DOPA high	124 ± 29	21 ± 5
LY (10)	L-DOPA high	159 ± 7	25 ± 3

* $p < 0.05$ versus Vehicle[a] + Vehicle[b], [a]empty oral capsule, [b]saline injection sc.
† Duration of the antiparkinsonian response expressed in minutes.
‡ Magnitude of the antiparkinsonian response expressed as the sum of points of improvement in the Disability Scale, accrued over a period of 4 hours following drug administration.

Dyskinesia response. CX516 given alone at 50 mg/kg produced dyskinesias in two of the eight animals (mean ± SEM, DSI 1.2 ± 0.7) for a mean duration of 17 ± 12 minutes, and at 100 mg/kg five animals exhibited dyskinetic movements (DSI 3.8 ± 1.6; figure 2B) for a mean duration of 48 ± 13 minutes. No dyskinesias were observed after any dose of LY300164 monotherapy (figure 2A).

A low dose of levodopa, either alone or with any dose of LY300164, produced no dyskinesias (see figure 2A). In contrast, the combination of low-dose levodopa with high-dose CX516 (100 mg/kg) generated dyskinesias in five animals

Table 2 Magnitude and duration of the antiparkinsonian response (means ± SEM) in eight levodopa-primed parkinsonian monkeys after treatment with levodopa (low-, medium-, or high-dose L-DOPA) alone or in combination with CX516 (CX)

Drug A, mg/kg	Drug B	Response duration†	Response magnitude‡
Vehicle[a]	Vehicle[a]	0	0
Vehicle[a]	L-DOPA low	0	2 ± 1*
Vehicle[a]	L-DOPA medium	118 ± 14*	19 ± 1*
Vehicle[a]	L-DOPA high	163 ± 3*	27 ± 3*
CX (5)	Vehicle[a]	0	0
CX (50)	Vehicle[a]	2 ± 2	0.7 ± 0.5
CX (100)	Vehicle[a]	2 ± 2	0
CX (5)	L-DOPA low	6 ± 4	2 ± 1
CX (50)	L-DOPA low	12 ± 7	4 ± 2
CX (100)	L-DOPA low	10 ± 8	3 ± 1
CX (5)	L-DOPA medium	104 ± 13	17 ± 2
CX (50)	L-DOPA medium	115 ± 11	22 ± 3
CX (100)	L-DOPA medium	105 ± 13	19 ± 2
CX (5)	L-DOPA high	136 ± 10	26 ± 3
CX (50)	L-DOPA high	148 ± 11	28 ± 4
CX (100)	L-DOPA high	136 ± 11	24 ± 2

* $p < 0.05$ versus Vehicle[a] + Vehicle[a], [a]saline injection sc.
† Duration of the antiparkinsonian response expressed in minutes.
‡ Magnitude of the antiparkinsonian response expressed as the sum of points of improvement in the Disability Scale, accrued over a period of 4 hours following drug administration.

(DSI 8.0 ± 2.5; $p < 0.01$; see figure 2B) for a duration of 48 ± 17 minutes.

Medium- and high-dose levodopa caused dyskinesia in all animals (see figure 2). Dyskinesias in four monkeys characteristically occurred as choreiform movements (largely limb), whereas the other four animals evidenced dystonic (mainly lower limb) posturing. The type of dyskinesia observed was consistent and animal-related and did not appear to reflect drug, dose, or parkinsonian severity. The abnormal movements induced by CX515 monotherapy, although mild, were identical in nature with those induced by levodopa.

Coadministration of medium-dose levodopa with higher dose LY300164 decreased DSI values by 18% after 5 mg/kg and 33% after 10 mg/kg (both $p < 0.05$; see figure 2A). This antidyskinetic effect was even more prominent when LY300164 was combined with high-dose levodopa, leading to DSI reductions of 29% after 1 mg/kg, 36% after 5 mg/kg, and 40% following a dose of 10 mg/kg (all $p < 0.05$; see figure 2A).

CX516, conversely, produced a dose-dependent increase in dyskinesias when coadministered with either medium- or high-dose levodopa. In combination with the medium dose of levodopa, CX516 increased DSI values by 35% after 50 mg/kg and 52% after the dose of 100 mg/kg (both $p < 0.05$; see figure 2B). In combination with high-dose levodopa, there was a DSI increase of 32% at a CX516 dose of 100 mg/kg ($p < 0.05$; see figure 2B).

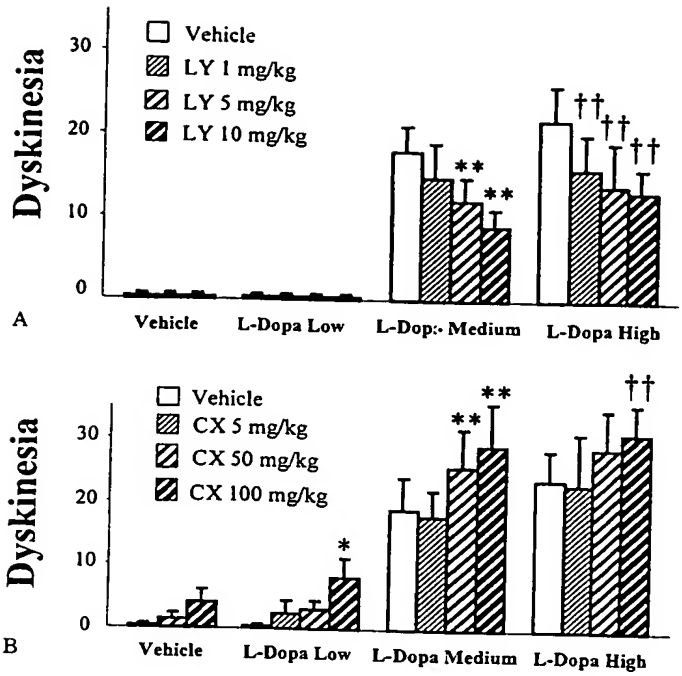

Figure 2. Dyskinesia, expressed as Dyskinesia Severity Index (DSI), following treatment with levodopa (low-, medium-, or high-dose L-DOPA) alone, or in combination with (A) LY300164 (LY) or (B) CX516 (CX). The index was derived from the formula:

(sum of all dyskinesia scores/duration of antiparkinsonian effect) × 100

*Values are mean ± SEM. *p < 0.05 versus L-DOPA low alone, **p < 0.05 versus L-DOPA medium alone, ††p < 0.05 versus L-DOPA high alone.*

Discussion. The predominant effects of the AMPA receptor agonist CX516 and the AMPA antagonist LY300164 on levodopa-induced dyskinesias were clearly demonstrated: CX516 monotherapy induced dyskinesias and potentiated those produced by levodopa, whereas LY300164 significantly attenuated levodopa-induced dyskinesias without worsening parkinsonian disability. There are no previous reports of the effects of AMPAergic drugs on levodopa-associated motor response alterations in parkinsonian primates. The current observations are, however, consistent with those found in parkinsonian rodents, where a competitive AMPA antagonist suppressed response alterations produced by chronic levodopa treatment[24] and reversed the neurochemical changes induced by nigrostriatal denervation in basal ganglia.[29] Relatively high doses of CX516 increased levodopa-induced dyskinesias by up to a 52%, whereas LY300164 reduced them by as much as 40%. Choreatic

and dystonic dyskinesias occurred with equal frequency and appeared to be animal- but not drug- or dose-specific.

AMPA antagonist administration also showed an antihypokinetic effect in MPTP-lesioned monkeys, albeit to a relatively modest degree. Moreover, high-dose LY300164 significantly potentiated the motor activating response of the group to low and medium doses of levodopa, as evidenced by significant changes in PAM counts. Concomitant changes could not be documented on the Disability Scale, presumably due to its relative insensitivity to minor degrees of improvement in moderately parkinsonian monkeys. The increase in PAM counts appears to reflect mainly an improvement in the antihypokinetic effects of levodopa; the amount of motor activity increased generally, although other parkinsonian signs were not obviously altered. Potentiation of the antiparkinsonian action of high-dose levodopa was not observed, presumably due to ceiling effects. The ampakine CX516 failed to modify the severity of the parkinsonian syndrome when given at any dose either alone or with levodopa.

Previous studies of AMPA antagonists in rodent and primate models have been limited to their antiparkinsonian activity. In 6-hydroxydopamine–lesioned or reserpine-treated rats, the AMPA antagonist NBQX had no effect, whereas it generally potentiated the motor effects of levodopa or DA agonists.[20-23,30,31] The only AMPA antagonist previously evaluated in MPTP-lesioned primates is NBQX. Although some antiparkinsonian activity was observed, the results are in partial conflict. In parkinsonian marmosets, NBQX as monotherapy failed to alter motor function but did potentiate the beneficial effect of a threshold dose of levodopa.[23] In rhesus monkeys, motor improvement was reported with monotherapy as well as a synergistic effect with levodopa coadministration.[30] In cynomolgus monkeys, however, NBQX had no activity alone and produced no synergism when coadministered with a D1 or D2 dopamine receptor agonist,[32] although the use of maximally effective dopamine agonist doses may have obscured any synergistic effect. These relatively disparate results could reflect different primate species, dissimilar MPTP lesion size, or the small number of animals studied, in combination with a relatively small effect size. Pharmacodynamic factors may also play a role. In the presence of excess glutamate, low doses of competitive antagonists such as NBQX may be ineffective, whereas larger doses produce unacceptable adverse effects. LY300164, in contrast, is a highly selective noncompetitive allosteric AMPA antagonist and therefore is less likely to be influenced by excessive glutamate concentrations. The findings of the current study indicate that LY300164 may exert some antiparkinsonian activity and thus appear generally consistent with the preponderance of previous observations.

The site of action of the AMPAergic drugs used in this study cannot be determined with the methodology employed. Glutamatergic receptors of both the NMDA and AMPA types are expressed in many brain regions, including cerebral cortex and all basal ganglia nuclei.[33] The most densely represented glutamate receptors in the striatum are those of the NMDA type, which seem to be largely if not exclusively expressed on the medium spiny neurons.[34] Thus it is not surprising that the ability of NMDA antagonists to reverse levodopa-induced response alterations in parkinsonian rats appears mainly attributable to effects in the striatum.[35] Regarding AMPA receptors, they too are richly expressed on the distal dendrites of striatal spiny neurons.[18] Conversely, early studies have suggested that AMPA receptors in the basal ganglia have a relatively higher density in the subthalamic nucleus (STN) and globus pallidus interna/substantia nigra pars reticulata (GPi/SNR),[16,36] although more recent results suggest a more even distribution.[19] Microinjection studies indicate, however, that AMPA has no effect in the striatum, but can induce parkinsonian signs when administered directly into the GPi/SNR or STN.[37] However, in our study the AMPA agonist CX516 did not apparently modify parkinsonian disability. Nonetheless, it produced dyskinesias, suggesting that dyskinetic and antiparkinsonian responses are mediated by distinct mechanisms. Conversely, whereas the infusion of NBQX into the GPi/SNR of reserpinized rats stimulates locomotion, it produces no effect when injected in the striatum.[38,39] Taken together, the available data provide limited support for the view that AMPA receptor-mediated synaptic mechanisms in the indirect pathway to the GPi/SNR play a crucial role in regulating motor function in parkinsonian models.

Whether mediated by AMPA receptors expressed on striatal efferents that project to the GPi, or on GPi efferents that project to the ventral lateral thalamus, it is important to know why AMPA receptor inhibition exerts antidyskinetic as well as relatively mild antihypokinetic activity. Some insight may derive from the results of NMDA antagonist studies. Evaluations in parkinsonian rodent and primate models suggest that the chronic nonphysiologic activation of striatal DA receptors, first as a consequence of denervation and later due to the intermittent high-intensity stimulation characteristically produced by dopaminomimetic therapy of these parkinsonian animals, activates certain signal transduction pathways in striatal medium spiny neurons.[5] These pathways, linking DA with nearby glutamatergic receptors, especially those of the NMDA type,[40] lead to the differential activation of kinases able to modify the phosphorylation state of NMDA receptor subunits.[7,8,41-43] As a result, the synaptic efficacy of NMDA receptor complex rises[44-47] and striatal output changes in ways that influence motor function.

The sensitivity of AMPA receptors is also affected by their phosphorylation state.[48,49] When upregulated, pharmacologic blockade would be expected to exert a normalizing effect, just as the NMDA antagonist amantadine tends to reduce both parkinsonian

signs and response alterations in parkinsonian models and patients.[11,14] The results of the current study would thus appear to be consistent with the possibility that AMPA receptors are also upregulated, possibly due in part to phosphorylation changes in association with the nigrostriatal system degeneration as well as subsequent intermittent levodopa treatment. Additional support for this possibility derives from our findings with CX516, a selective positive modulator of AMPA receptors, which exerted the opposite effects of LY300164 by strongly augmenting the dyskinesiogenic action of levodopa.

No completely satisfactory medical treatment for PD exists. A more comprehensive view of the reactive changes occurring in basal ganglia structures downstream from the dopamine system in parkinsonian patients might provide leads to the development of novel therapeutic strategies that will successfully palliate symptoms without the risk of the disabling adverse effects associated with current interventions. Indeed, focusing more on the secondary changes occurring in the striatum and downstream basal ganglionic structures may provide a good start in this direction. Therapeutic exploitation of recent discoveries concerning NMDA receptor changes has already proved useful in parkinsonian patients.[11] AMPA antagonists may prove no less useful clinically, as LY300164 produced a mild increase in general motor activity both when given alone and with low-dose levodopa but substantially blunted the dyskinesias induced by higher doses of levodopa without worsening parkinsonian disability. Control of peak dose dyskinesias with LY300164 could allow increases in levodopa dosage to improve "on" time in patients with "on–off" fluctuations. Conceivably, the pharmacologic targeting of basal ganglionic glutamatergic mechanisms modified in PD—initially as a consequence of dopaminergic denervation and later due to pulsatile dopaminergic stimulation—will stimulate a broader approach to medical management that could ultimately yield safer and more effective therapies for all stages of this disorder.

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AMPA Receptor–Mediated Neurotoxicity: Role of Ca^{2+} and Desensitization*

A Frandsen[1] and Arne Schousboe[1,2]

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Glutamate-induced neurodegeneration is the result of excessive stimulation of the different subtypes of glutamate receptors. With regard to the AMPA ((RS)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate) receptors it has been clear from numerous studies that in addition to the Ca^{2+} permeability of the receptor complexes, their desensitization properties may play a determining role in the neurodegeneration mediated by this subtype of the glutamate receptors. Recent studies have revealed important amino acid residues in the AMPA receptor subunits that control the desensitization kinetics and that may constitute important targets for drugs that may alter the desensitization of the AMPA receptor complexes.

KEY WORDS: Neurons; excitotoxicity; glutamate; kainate; AMPA; desensitization.

TRODUCTION

It has been recognized for about half of a century that activation of the glutamatergic neurotransmission system under certain conditions may lead to degeneration of nerve cells (1,2), a process termed excitotoxicity. At the time when solid pharmacological and electrophysiological evidence had firmly implicated glutamate receptor activation in the glutamate neurotransmission process (3) it was clear that the glutamate induced neurodegeneration was intimately related to activation of glutamate receptors (4). Pharmacological studies aimed at characterizing this phenomenon in combination with the cloning of the different types of ionotropic glutamate receptors have led to the conclusion that the excitotoxic process can be initiated by activation of each one of the three types of ionotropic glutamate receptors, the *N*-methyl-D-aspartate-(NMDA), AMPA, and kainate receptors (5,6). The present short review focuses on a discussion of the role of the AMPA receptors in this context. Since the discovery of the differential Ca^{2+} permeability of different populations of AMPA receptor complexes (7) it has been extensively debated as to what extent the Ca^{2+} permeability of these receptors may regulate the excitotoxic process (8,9). More recently it has become evident that in addition to the Ca^{2+} permeability, the differential desensitization kinetics of AMPA receptor complexes may play an important role in the excitotoxic process (10,11). The review focuses on these aspects of AMPA receptor–mediated neurotoxicity.

DISCUSSION

AMPA Receptors

AMPA receptor complexes are composed of four subunits that form an ion channel permeable to sodium ions when activated by binding of the agonist (7). Electrophysiological studies of recombinantly expressed receptor complexes containing different combinations of

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[1] Department of Pharmacology, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100, Copenhagen, Denmark.
[2] Address reprint requests to: Department of Pharmacology, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100, Copenhagen, Denmark. Tel: +45 35306330; Fax: +45 3536021; E-mail: as@mail.dfh.dk

the four receptor subunits (GluR1-4) have revealed an important role of the GluR2 subunit with regard to the ionic selectivity of the channel (12). Thus it has been firmly established that this subunit, if present in the receptor complex, confers a pronounced Na^+ selectivity of the channel. However, if the receptor complexes are formed without the GluR2 subunit, the resulting ion channel is permeable not only to Na^+ but also to Ca^{2+} (13). This property of the GluR2 subunit is based on an editing process leading to the existence of two GluR2 subunits with either an arginine or a glutamine residue at position 607 in the second transmembrane loop (13). The arginine-containing subunit that in vivo is predominant confers the sodium selectivity to the resulting receptor complex (13). This obviously has profound functional consequences given the fact that intracellular Ca^{2+} homeostasis controls a large variety of cellular functions (14).

In addition to the above mentioned differential Ca^{2+} permeability of AMPA receptor complexes consisting of different subunit compositions, such receptor complexes may exhibit different desensitization kinetics. As illustrated in Fig. 1, homomeric GluR1 and GluR3 receptors expressed in *Xenopus laevis* oocytes exhibit a pronounced difference in the desensitization kinetics (15). Thus the desensitization rate constant (τ) for the $GluR1_0$ complex is about 4 ms, while that for $GluR3_0$ or $GluR4_0$ complexes is about 1 ms. As shown by Lomeli et al. (16) this difference in desensitization kinetics may be related to the amino acid residue at position 757, which in $GluR1_0$ is an arginine, whereas at the corresponding position in $GluR3_0$ a glycine is found. This is normally referred to as the R/G editing site and is thought to play a significant role in the desensitization mechanism (16). The AMPA receptor subunits exist in two splice variants termed the flip/flop forms (17), which greatly influence the desensitization kinetics. Thus the flop variant exhibits much faster desensitization kinetics than the flip forms (18). It is noteworthy that during postnatal development the expression of the two splice variants undergoes a change toward a more pronounced expression of the flop variant at more mature stages (19). The observation that AMPA receptors desensitize has led to the discovery of the mechanism of action of a number of drugs including cyclothiazide that block the desensitization of these receptors (20,21). A recent study of the ligand binding domain of the S1–S2 region of the GluR1 and GluR3 AMPA receptor subunits using the AMPA analog bromohomoibotonic acid (BrHIBO) and chemical modeling has provided additional information concerning amino acid recidues that may play an important role with regard to the desensitization process (15). Inspection of a critical region of the S2 segment of the extracellular

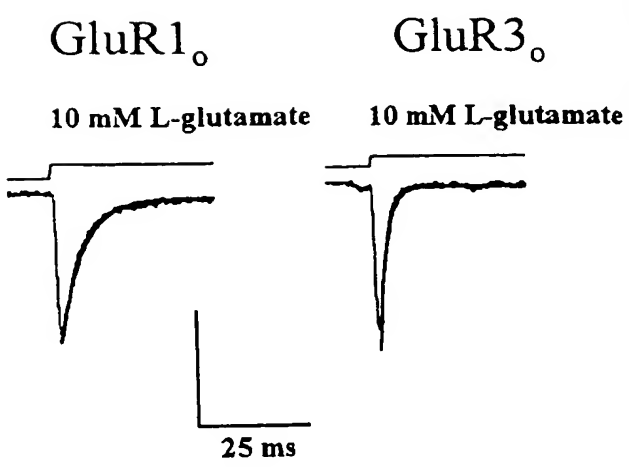

Fig. 1. Homomeric $GluR1_0$ or $GluR3_0$ receptor complexes were expressed in *Xenopus laevis* oocytes (15), and currents were subsequently recorded from outside-out patches using fast recording and application of the ligand (L-glutamate) employing a piezoelectric element (15). Briefly, injected oocytes (50–100 nl of ~1 µl cRNA) were prescreened with a two-electrode voltage clamp; those having a response >200 nA (V_h = −80 mV) to 300 µM KA were selected for further investigation. The vitelline membrane was removed by placing the oocyte in a 35-mm dish containing a hyperosmotic medium (in mM): 200 K^+ aspartate, 20 KCl, 1 $MgCl_2$, 5 EGTA-KOH, and 10 HEPES-KOH, pH 7.4. After 10–15 min in the solution, the vitelline membrane was removed with a pair of fine forceps. Outside-out patches from oocytes were prepared with thin-walled glass capillaries (World Precision Instruments, Sarasota, FL, USA) filled with (in mM): 100 KCl, 10 EGTA, and 10 HEPES, pH 7.0. Pipettes had a resistance of 3–5 MΩ. The external solution was frog Ringer's. Fast application of agonists to outside-out membrane patches was made using a double-barreled theta glass tube (outer diameter, 2.0 mm; wall thickness, 0.3 mm; septum thickness, 0.12 mm; Hilgenberg, Malsfeld, Germany). Frog Ringer's solution flowed continuously through one barrel, while the other barrel contained 10 mM L-glutamate. The graph shows the currents (scale bar = 60 pA) elicited by application of 10 mM L-glutamate as indicated above the current trace. (From Banke et al. [15] with permission).

loop between TM3 and TM4 of GluR1 and GluR3 reveals that 15 amino acids are different from each other (15). An investigation of τ for homomeric receptors consisting of GluR1 subunits in which each one of these amino acid residues were mutated to the amino acids present in the corresponding position of the GluR3 subunits revealed a crucial tyrosine residue in position 716 of the GluR1 that is replaced by a phenylalanine residue of GluR3. Molecular modeling has shown that the hydroxyl group of the tyrosine is critically involved in hydrogen binding of a water molecule that interacts with the agonist, thus influencing the binding and desensitization characteristics of the receptor complexes. It may be concluded that desensitization is not only controlled by the residue 757 (arginine/glycine) but also by residue 716 (tyrosine/phenylalanine), as shown by Banke et al. (15).

AMPA Toxicity

Numerous studies of the pharmacological characteristics of glutamate induced cytotoxicity in a variety of neuronal phenotypes have provided evidence to suggest that activation of all subtypes of ionotropic glutamate receptors can lead to neuronal damage (4,9,22). Hence, activation of AMPA receptors is of importance, although it should be emphasized that indirect involvement of NMDA receptors activated by released endogenous glutamate should always be controlled for by application of an NMDA receptor antagonist together with AMPA (10,23,24). The fact that AMPA receptor activation plays an important role in this context has stimulated investigations aimed at elucidating the roles of the Ca^{2+} permeability of the AMPA receptors as well as their desensitization characteristics.

Role of Ca^{2+} Permeability. While there is general consensus regarding the notion that aberrations in the intracellular Ca^{2+} homeostasis will lead to neuronal damage and cell death (e.g., [8]) it may be less clear what may be the exact role of the AMPA receptors. Several studies have provided evidence to suggest that Ca^{2+} influx through AMPA receptors may play a role in AMPA-mediated neuronal degeneration (25,26). This notion received considerable interest by the demonstration that subsequent to an is chemic episode leading considerable neuronal damage in the CA1 region of hippocampus, there was a significant downregulation of the expression of the GluR2 AMPA receptor subunit, thus increasing the Ca^{2+} permeability of the resulting receptor complexes (27). This GluR2 hypothesis has subsequently been questioned by others who have failed to reproduce this finding (28). In this context it may be of interest that no simple correlation could be demonstrated in cerebral cortical neurons in culture between the number of neurons expressing Ca^{2+}-permeable AMPA receptors and the relative abundance of the GluR2 subunit compared to the other AMPA receptor subunits (29,30). Moreover, the relative expression of the GluR2 subunit as a function of development in culture was found not to change significantly (30), whereas the sensitivity of these cultured neurons to AMPA or kainate exposure has been shown to increase with the culture period (31). This underlines the complex interrelationship between AMPA neurotoxicity and expression of Ca^{2+}-permeable AMPA receptors, as also noted by others (11).

Role of Desensitization. Studies of the functional role of AMPA receptor desensitization have been greatly facilitated by the introduction of drugs such as cyclothiazide, which by binding to a specific site in the receptor complex block the desensitization of the AMPA receptors (20,21,32). Using this tool it has been shown that the desensitization property of the AMPA receptor complex is of importance for its Ca^{2+} permeability (11,29,30,33,34). Thus the Ca^{2+} influx through Ca^{2+}-permeable AMPA receptors is greatly enhanced in cerebral cortical neurons by pretreatment with cyclothiazide (29). This would indicate that the desensitization property of the AMPA receptors might play an important role in the AMPA-receptor mediated neurotoxicity. Indeed, as shown by Brorson et al. (35) the desensitization of AMPA receptors predicts the sensitivity of neurons to excitotoxicity. In keeping with this, a number of studies have unequivocally demonstrated that blocking the desensitization of AMPA receptors using cyclothiazide leads to a pronounced enhancement of AMPA-mediated neuronal degeneration (10,11,30,36,37). Hence, it appears that this property of the AMPA receptors may be of significant importance in controlling a possible involvement of these receptors in events leading to neurodegeneration. This notion may be underlined by the recent demonstration that CPW399, a nondesensitizing selective AMPA receptor agonist exhibits pronounced neurotoxicity that is independent of cyclothiazide (38).

CONCLUSION

The above discussion has revealed a certain mismatch between the ability of AMPA receptors to gate Ca^{2+} influx and their role in the excitotoxic process. On the contrary, it seems well supported that a much better correlation exists between the ability of AMPA receptors to control desensitization and their ability to mediate a neurotoxic signal. As a matter of fact it may be concluded that the desensitization process may be the governing property of AMPA receptors controlling Ca^{2+} permeability and their involvement in neurotoxicity mediated by excitatory amino acids. Given the fact that excitotoxicity plays a prominent role in relation to neurodegeneration triggered by energy failure (e.g., ischemia), as well as in a number of neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, ALS, and Huntington's chorea) it may be of therapeutic relevance to intensify the development of tools by which the desensitization of AMPA receptors may be manipulated. The relatively detailed knowledge available concerning the molecular basis for the desensitization process may facilitate such studies.

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New Non Competitive AMPA Antagonists

Gizella Ábrahám, Sándor Sólyom,* Emese Csuzdi, Pál Berzsenyi, István Ling,[†] István Tarnawa,[‡] Tamás Hámori, István Pallagi, Katalin Horváth,[§] Ferenc Andrási, Gábor Kapus, László G. Hársing Jr., István Király, Miklós Patthy and Gyula Horváth

Institute for Drug Research, Ltd, Berlini-u. 47-49, H-1045 Budapest, Hungary

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Abstract—New halogen atom substituted 2,3-benzodiazepine derivatives condensed with an azole ring on the seven membered part of the ring system of type **3** and **4** as well as **5** and **6** were synthesized. It was found that chloro-, dichloro- and bromo-substitutions in the benzene ring and additionally imidazole ring condensation on the diazepine ring can successfully substitute the methylenedioxy group in the well known molecules GYKI 52466 (**1**) and GYKI 53773 (**2**) and the 3-acetyl-4-methyl structural feature in **2**, respectively, preserving the highly active AMPA antagonist characteristic of the original molecules. From the most active compounds (**3b,i**) **3b** (GYKI 47261) was chosen for detailed investigations. **3b** revealed an excellent, broad spectrum anticonvulsant activity against seizures evoked by electroshock and different chemoconvulsive agents indicating a possible antiepileptic efficacy. **3b** was found to be highly active in a transient model of focal ischemia predictive of a therapeutic value in human stroke. **3b** also reversed the dopamine depleting effect of MPTP and antagonized the oxotremorine induced tremor in mice indicating a potential antiparkinson activity.

Introduction

Excessive activation of the glutamate receptors may be a major factor in the pathologenesis of several acute and chronic neurological disorders.[1] Attention has been focused to identify selective antagonists according to the types of the ionotropic glutamate receptors classified according to their exogenous ligands *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-methylisoxazol-4-yl)propionic acid (AMPA) and kainic acid.[1c] Selective AMPA antagonists have gained special importance recently.[2] Among them the noncompetitive antagonists exerting their effect at an allosteric site of the receptor are of particular interest because of the theoretical possibility that they are effective even at extraordinarily high levels of the natural transmitter glutamate, as well.[3] Some of the potential therapeutic targets of these antagonists may be epilepsy, spasticity, pain, and neurodegenerative disorders.[3]

The 2,3-benzodiazepine derivative GYKI 52466 (**1**) was the prototype of the noncompetitive AMPA antagonists[4] (Fig. 1). From our structure–activity relationship study GYKI 53773 (**2**, LY300164, talampanel), a highly active AMPA antagonist emerged from the 3-acylated 3,4-dihydro analogues of **1**,[5] which is now one of the clinically most advanced agents among the non competive AMPA antagonists.[6] Further structure–activity relationship studies revealed several structural features which are important to maintain the potent AMPA antagonistic character of the original molecules **1** and **2**.[7] We have found that halogen atoms in the benzene ring can successfully substitute the dioxolane ring in **1** and **2**[7d] and the biological activity was also retained when the 3-acyl-4-methyl substitution pattern in **2** was replaced by some nitrogen containing heterocycles attached to the 3,4-positions of the 2,3-benzodiazepine ring system.[7b] In this paper we report the synthesis and preliminary pharmacological studies of compounds **3**–**6**, where halogen atom(s) and condensed nitrogen containing five membered heterocycles were simultaneously applied to the 2,3-benzodiazepine ring system.[7e]

Chemistry Results

One of the key steps in the synthesis of new 2,3-benzodiazepines with halogen atoms in the benzene ring is the

*Corresponding author. Tel.: +36-1-399-3471; fax: +36-1-399-3356; e-mail: h13759sol@ella.hu
[†]Recent address: EGIS, Kereszturi út 30-38, Budapest, Hungary.
[‡]Gedeon Richter Ltd, Gyömrői-u. 19-21, H-1103 Budapest, Hungary.
[§]Pharmaceutical Control and Development Laboratory, Mexikói út 9, H-1149 Budapest, Hungary.
[¶]A subsidiary of IVAX Corporation, Miami, Florida, USA.

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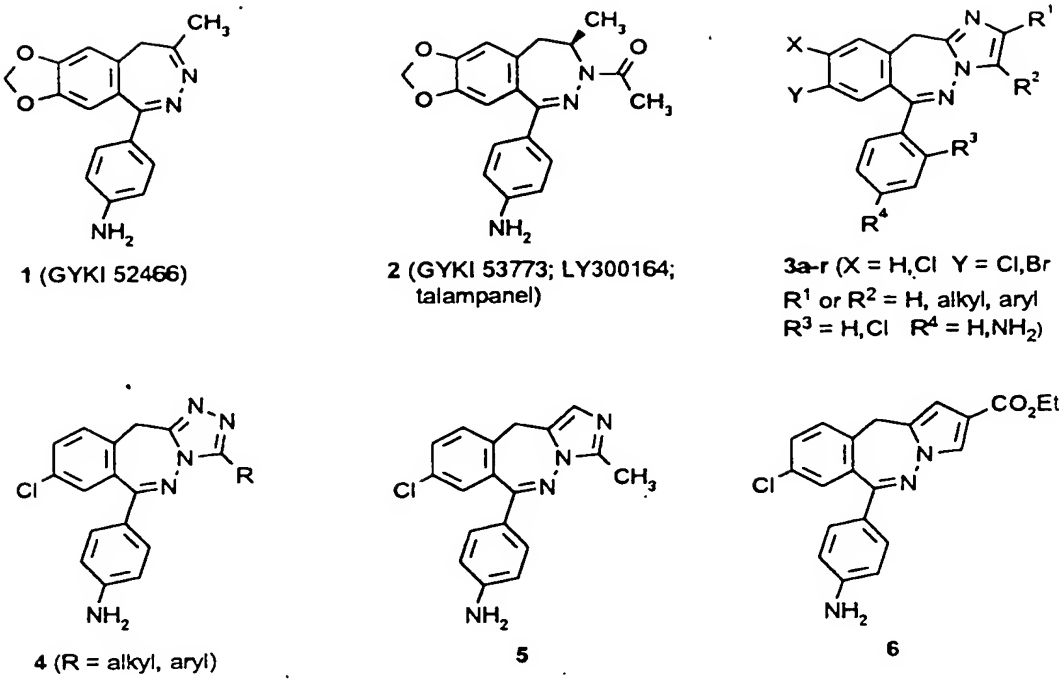


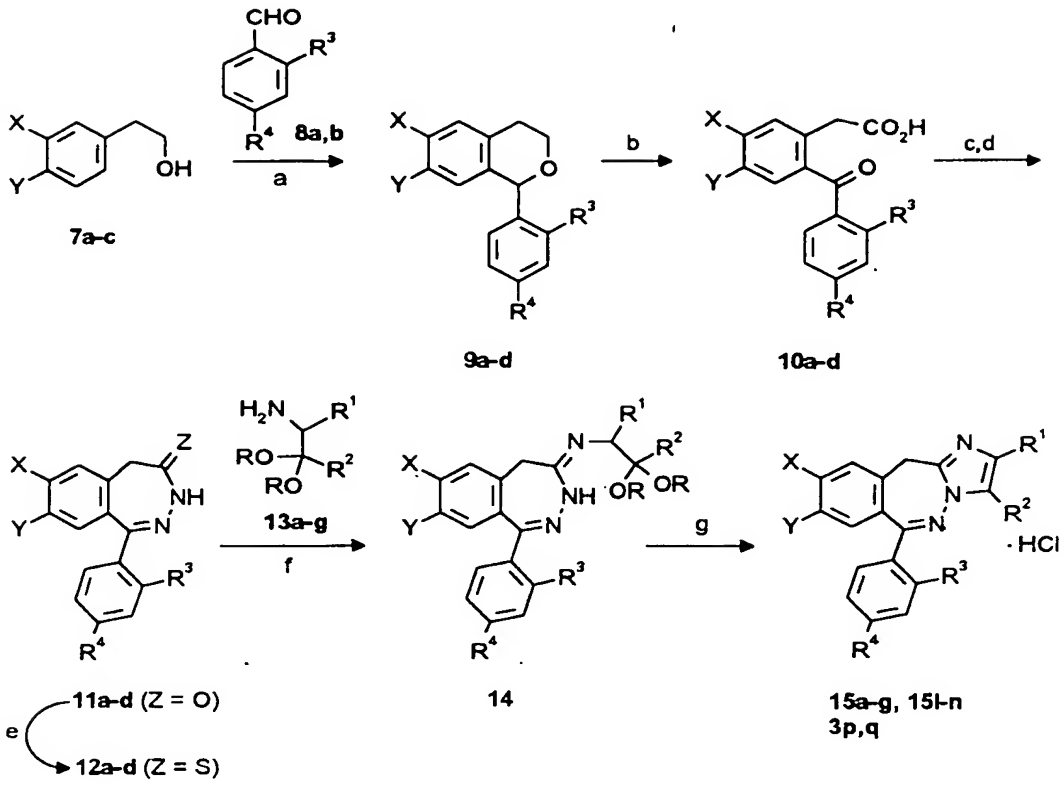
Figure 1.

formation of isochromanes **9a–d** from the corresponding alcohols and an aldehyde (Scheme 1). While the same reaction can be easily performed in the case of alcohols with electron donating groups, like, e.g. with 2-(3,4-dimethoxyphenyl)- or 2-(3,4-methylenedioxyphenyl)ethanol or the corresponding isopropanols and 4-nitrobenzaldehyde using only slightly more than one equivalent of concd hydrochloric acid,[8] the analogous reactions with **7a–c** need anhydrous conditions in benzene using freshly molten zinc chloride and dry hydrogen chloride gas and even then the yields are moderate. But on the other hand the enhanced electrophilicity of the reacting benzaldehyde derivative must contribute to the success of the reaction as well, since, e.g. under the same reaction conditions only negligible reaction could be noticed between **7a** and unsubstituted benzaldehyde. It was also found e.g. with the less electrophilic **8b** that apart from the unknown steric contribution of the *ortho* substituent to the reaction the corresponding isochromane **9d** formed with significantly lower yield than the analogous **9b** in the reaction between **7b** and **8a**.

The reaction of isochromanes **9a–d** with Jones reagent in acetone provided ketocarboxylic acids **10a–d**. The latter were reacted with excess hydrazine hydrate in ethanol to give the intermediate hydrazones which were treated in turn with dicyclohexylcarbodiimide to achieve ring closure. Another possibility to induce ring closing reaction was the treatment of the intermediate hydrazone with excess hydrochloric acid for longer time to provide the 4-oxo-2,3-benzodiazepine derivative, e.g. **11a**.

In order to enhance the reactivity of the 4-oxo compounds **11a–d** towards condensation reactions to build up the expected imidazolo- or triazolo-2,3-benzodiazepines, we have chosen the corresponding thiooxo derivatives **12a–d**, which were prepared from **11a–d** by phosphorous pentasulfide in dry pyridine with acceptable yields.[9] The latter were reacted further with the known aminoacetals **13a–g**. This condensation step was most successful when the reactants were heated in 2-methoxyethanol using red mercury oxide as the sulfur binding agent. The resulting intermediates of type **14** were generally purified by column chromatography and then further reacted with hydrochloric acid to give the imidazolo compounds **15a–g** and **15i–n** as well as **3p,q**. respectively. The nitro groups in benzodiazepines **15a–n** were reduced by a standard method using hydrazine hydrate and RaNi to give products **3a–n**.[10] Because of solubility considerations the reductions were carried out generally in a mixture of methanol and dichloromethane. As imidazolo-benzodiazepines with unsubstituted phenyl ring can hardly be synthesized by the above route, because of the difficulties concerning the corresponding isochromane formation, this type of compound was prepared by removal of the amino group, e.g. in **3c** by reaction with isoamyl nitrite and 5n hydrochloric acid to provide **3r**.[11] In one instance the aromatic amino group of **3b** was acetylated to give **3o** as one of the potential metabolites. The prepared imidazolo compounds are listed in Table 1.

When **3b** as one of the biologically most interesting compounds had to be prepared in multigram scale we became interested to avoid some disadvantages of the synthetic route outlined in Scheme 1. These were the 5 step synthesis of the acetal **13b** from alanine and the optional but useful column chromatography of the intermediates of type **14**. It was attractive to make use of a relatively scarcely used 4 + 1 cyclization method to prepare the imidazole ring, where an acylamidoketone is



7	a	b	c
X	H	H	Cl
Y	Cl	Br	Cl

8	a	b
R^3	H	Cl
R^4	NO_2	H

9-12	a	b	c	d
X	H	H	Cl	H
Y	Cl	Br	Cl	Br
R^3	H	H	H	Cl
R^4	NO_2	NO_2	NO_2	H

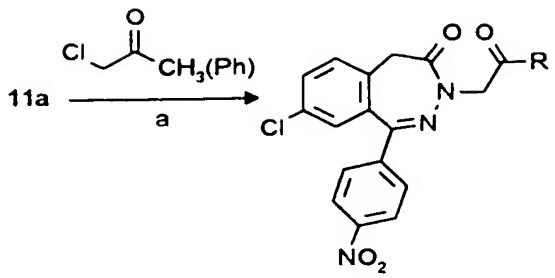
13	a	b	c	d	e	f	g
R^1	H	Me	H	Me	Et	H	H
R^2	H	H	Me	Me	H	4-NO_2-C_6H_4-	4-pyridyl
R+R	CH_2CH_2	CH_2CH_2	CH_2CH_2	CH_2CH_2	CH_2CH_2	Et, Et	Et, Et

3/15	a	b	c	d	e	f	g
X	H	H	H	H	H	H	H
Y	Cl	Cl	Cl	Cl	Cl	Cl	Cl
R^1	H	Me	H	Me	Et	H	H
R^2	H	H	Me	Me	H	4-NH_2-C_6H_4- /4-NO_2-C_6H_4-	4-pyridyl
R^3	H	H	H	H	H	H	H
R^4	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2

3/15	i	j	k	l	m	n
X	Cl	Cl	H	H	H	H
Y	Cl	Cl	Br	Br	Br	Br
R^1	Me	H	Me	H	Me	H
R^2	H	Me	H	Me	Me	4-pyridyl
R^3	H	H	H	H	H	H
R^4	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2	NH_2/NO_2

3	o	p	q	r
X	H	H	H	H
Y	Cl	Br	Br	Cl
R^1	Me	Me	H	H
R^2	H	H	Me	Me
R^3	H	Cl	Cl	H
R^4	NHCOMe	H	H	H

Scheme 1. Route I. (a) benzene, $ZnCl_2$, HCl(g); (b) acetone, CrO_3/H_2SO_4; (c) EtOH, $H_2NNH_2{\cdot}H_2O$, Δ; (d) DCC or HCl; (e) pyridine, P_2S_5, 80 °C; (f) 2-methoxyethanol, red HgO, Δ; (g) HCl, Δ; (h) MeOH-CH_2CL_2, RaNi, $H_2NNH_2{\cdot}H_2O$; (i) DMF, isoamylnitrite, 65 °C; (j) pyridine/AcCl, 5 °C.



b → 15b,h

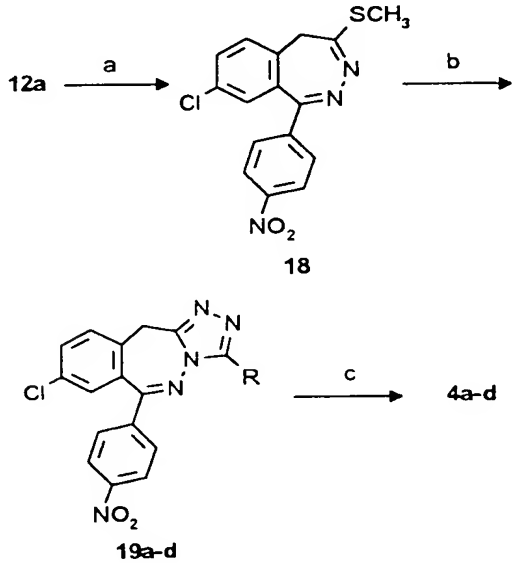
15	b	h
X	H	H
Y	Cl	Cl
R^1	Me	Ph
R^2	H	H
R^3	H	H
R^4	NO_2	NO_2

Scheme 2. Route II. (a) DMF, K_2CO_3; (b) NH_4OAc, AcOH, Δ.

reacted with ammonium acetate as nitrogen atom donor in acetic acid.[12] The necessary amidoketone **16** was prepared in very good yield by alkylation of **11a** with chloroacetone (Scheme 2). We have found that condensation of **16** to give the corresponding imidazolo-benzodiazepine **15b** needs a large excess (>50 equivalents) of ammonium acetate in acetic acid and the yield is modest (40%). The use of other ammonium salts like formate or carbonate as well as solvents, e.g. formamide, propionic acid, trifluoroacetic acid or microwave techniques instead of conventional heating did not give significant improvement. Despite the lower yield of the imidazole forming step, **3b** can be produced by this route without any laborious synthetic steps. We attribute the lower yield of this condensation step to the sensitive nature of the acetone side chain in **16** towards reactions, since with the phenacyl derivative **17** the same condensation could be performed with a significantly better yield (82%). (Scheme 2.)

For the formation of the triazolo ring in compounds **4a–d** acylhydrazides were used (Scheme 3).[13] Better results could be achieved in the condensation steps when instead of thione **12a** the corresponding 4-methylthio-2,3-benzodiazepine **18** was used and additionally a catalytic amount of hydrochloric acid was applied, as well. The nitro groups in compounds **19a–d** were reduced by the standard RaNi-hydrazine hydrate method and the resulting compounds **4a–d** are shown in Table 2.

To build up the 11*H*-imidazolo[3,4-c][2,3]benzodiazepine ring system in **5** we started from **23** which was synthesized on the analogy to well established methods (Scheme 4).[14] The methyl group in **23** was oxidized by selenium dioxide to form the corresponding aldehyde **24**, which was then reduced to the alcohol **25** by sodium borohydride. The



4/19	a	b	c	d
R	Me	4-pyridyl	4-NH_2-C_6H_4- /4-NO_2-C_6H_4-	CH_3O-CH_2-

Scheme 3. (a) acetone, MeJ, K_2CO_3; (b) DMF, acylhydrazine, cat. HCl; (c) MeOH-CH_2Cl_2, RaNi, H_2NNH_2-H_2O.

Table 1. 11H-Imidazolo[1,2-c][2,3]benzodiazepine derivatives (3a–r)

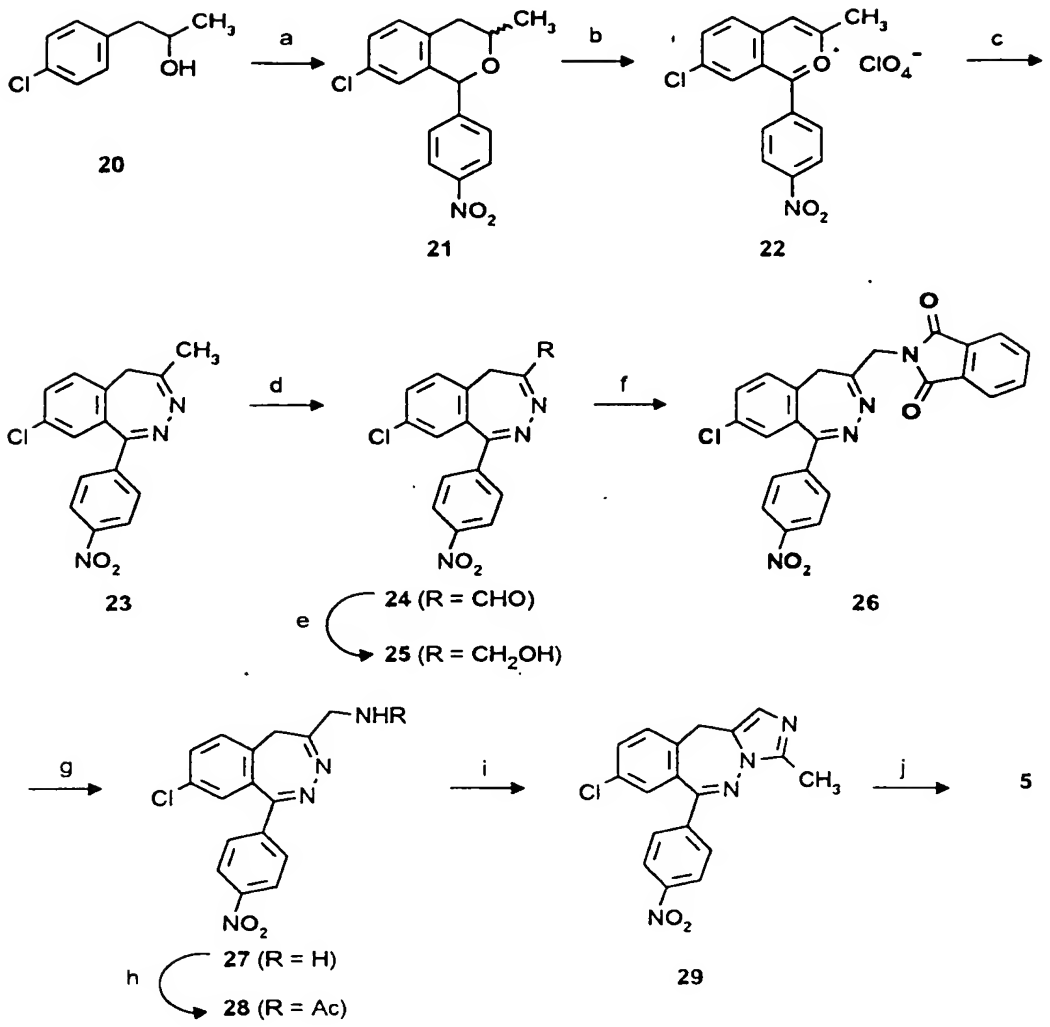
Compound	X	Y	R^1	R^2	R^3	R^4	mp (°C)	Yield (%)[a]
3a	H	Cl	H	H	H	NH_2	210–214	67
3b	H	Cl	Me	H	H	NH_2	229–230	79
3c	H	Cl	H	Me	H	NH_2	267–270	71
3d	H	Cl	Me	Me	H	NH_2	274–278	83
3e	H	Cl	Et	H	H	NH_2	247–250	72
3f	H	Cl	H	4-NH_2-C_6H_4—	H	NH_2	250–253	84
3g	H	Cl	H	4-pyridyl	H	NH_2	293–294[b]	68
3h	H	Cl	Ph	H	H	NH_2	223–226	81
3i	Cl	Cl	Me	H	H	NH_2	254–255	40
3j	Cl	Cl	H	Me	H	NH_2	284–286	71
3k	H	Br	Me	H	H	NH_2	248–251	64
3l	H	Br	H	Me	H	NH_2	263–268	77
3m	H	Br	Me	Me	H	NH_2	272–275	84
3n	H	Br	H	4-pyridyl	H	NH_2	295–300[b]	41
3o	H	Cl	Me	H	H	NHCOMe	265–266	63
3p	H	Br	Me	H	Cl	H	132–140[c]	21
3q	H	Br	H	Me	Cl	H	210–215[c]	48
3r	H	Cl	H	Me	H	H	166–169	39

[a]Yields refer to the last step of the synthesis.
[b]Decomposition.
[c]Hydrochloride salts.

Table 2. 6-(4-Aminophenyl)-8-chloro-11H-1,2,4-triazolo[4,5-c][2,3]benzodiazepine derivatives (4a–d)

Compound	R	mp (°C)	Yield (%)[a]
4a	Me	228–231	91
4b	4-pyridyl	284–288	92
4c	4-NH_2-C_6H_4—	191–193	85
4d	CH_3OCH_2—	195–197	83

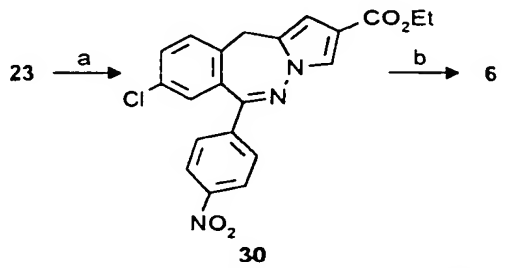
[a]Yields refer to the last step of the synthesis.



Scheme 4. (a) 4-nitrobenzaldehyde, benzene, $ZnCl_2$, HCl(g); (b) acetone, CrO_3/H_2SO_4, then 70% $HClO_4$; (c) DMF, $H_2NNH_2{\cdot}H_2O$; (d) dioxane, SeO_2; (e) THF-H_2O, $NaBH_4$; (f) THF, Ph_3P, phthalimid, DEAD; (g) MeOH, $H_2NNH_2{\cdot}H_2O$; (h) Ac_2O; (i) $ClCH_2CH_2Cl$, $POCl_3$, Δ; (j) MeOH-CH_2Cl_2, RaNi, $H_2NNH_2{\cdot}H_2O$.

latter was transformed under Mitsunobu conditions[15] with phthalimide into **26** then hydrazinolysis and acetylation gave **27** and **28**, respectively. Cyclization of **28** with phosphorus oxychloride gave **29** which was reduced by RaNi-hydrazine hydrate to give **5**.

The pyrrolo derivative **6** was prepared from **23** by using a combined alkylation-condensation procedure[16] with ethyl bromopyruvate to give the nitro compound **30** which on reduction provided **6** (Scheme 5).



Scheme 5. (a) Br-CH_2-C(O)-CO_2Et, EtOH, Δ; (b) MeOH-CH_2Cl_2, RaNi, $H_2NNH_2{\cdot}H_2O$.

Biological Results and Discussion

Primary pharmacological testing and structure–activity relationships

Table 3 contains primary in vitro and in vivo biological data with the newly synthesized compounds in comparison with the standard molecules **1** (GYKI 52466) and **2** (GYKI 53773, LY300164, talampanel).

Inhibition of AMPA-, or kainate-triggered spreading depression in isolated chicken retina. Excitation of isolated chicken retinas by glutamate receptor agonists provokes spreading depression (SD), which is accompanied by a characteristic change of the light scattering properties of the preparation. The phenomenon is easily visible by unaided eye, thus it is a convenient method for drug

Table 3. Screening results of the condensed 2,3-benzodiazepine derivatives 3–6[a]

Compond	Behavioural changes (100 mg/kg ip; 200 mg/kg po)	Retinal spreading depr. IC_{50} (μM)	MES ED_{50}, mg/kg po	Inc. screen ED_{50}, mg/kg ip
1	Loss of righting reflex	A: 6.3 K: 9.5	37.4	47.1
2	Loss of righting reflex	A: 1.7 K: 2.6	8.6	13.4
3a	Short loss of righting reflex	A: 6.5 K: 3.8	61.6	47.1
3b	Loss of righting reflex	A: 7.3 K: 2.5	24.0	36.5
3c	Ataxia, muscle relaxation, po: loss of righting reflex	A: 4.1 K: 0.5 (flat curve)	61.3	100-125
3d	Ataxia	A: 11.8 K: 1.5	~100	> 200
3e	Loss of righting reflex, ataxia, SMA↓	A: > 20 K: > 20	44.6	> 150
3f	SMA↓, ataxia	A: 4.3 K: 7.1	> 100	~200
3g	SMA↓, ataxia	A: 3.1 K: n.t.	> 100	> 200
3h	Ø	A: > 20 K: n.t.	> 100	> 200
3i	Loss of righting reflex	A: 3.1 K: 1.9	24.0	47.2
3j	po: SMA↓, ataxia	A: > 20 (0.6–10 μM: max. 47% inhibition) K: 1.1	50–100	> 200
3k	Loss of righting reflex	A: 9.3 K: 2.2	40.3	68.4
3l	SMA↓, ataxia, weak muscle relaxation	A: 4.0 K: 1.3	~100	~200
3m	SMA↓, ataxia	A: > 20 K: 7.4	~100	> 200
3n	po: SMA↓	A: 3.2 K: > 20	> 100	> 200
3o	SMA↓, ataxia	A: > 20 K: > 20	56.0	> 200
3p	ip: Weak SMA↓	A: > 20 K: > 20	> 100	> 200
3q	ip: SMA↓	A: > 20 K: > 20	> 100	~200
3r	ip: Weak SMA↓	A: > 20 K: > 20	> 100	> 200
4a	Ataxia, weak muscle relaxation	A: 2.9 K: 4.1	~100	~100
4b	Ø	A: > 20 K: ~20	> 100	> 200
4c	Ø	A: > 20 K: 1.0	> 100	> 200
4d	SMA↓, ataxia	A: 9.2 K: 5–10	> 100	~150
5	ip: SMA↓, ataxia	A: > 20 K: n.t.	~100	> 200
6	100 ip.: Ø	A: > 20 K: n.t.	> 100	~200

[a]SMA↓: decrease of spontaneous motor activity; A: AMPA; K: kainate; n.t.: not tested.

testing. Retinal SD can be blocked by glutamate antagonists.[17] In our study the majority of the tested compounds were effective in the retinal spreading depression (SD) test, i.e. blocked either AMPA- or kainate induced SD with an IC_{50} lower than 10 μM. The IC_{50} values of the most potent compounds were similar to those of **1** or **2** in the AMPA test, and lower in the kainate test. Compounds **3b,d,j,k,l,m** seemed to show some selectivity towards kainate responses, i.e. their IC_{50} values were more than 2-fold higher against AMPA than against kainate. The concentration-response curves of **3b** against the two agonists are shown in Fig 2. Interestingly, some compounds such as **3e**, **3m**, or **4d** caused only partial inhibition of kainate-triggered SD (maximal inhibition: 40–60% at 20 μM; not shown), or had a flat dose-response curve (**3a**, **3c**, or **3i**; not illustrated). The significance of the differences between AMPA and kainate antagonistic actions in the case of some compounds is difficult to tell, as the role of the specific kainate receptors in triggering SD in this preparation has not been characterized yet. It is to be noted here that the competitive AMPA antagonist 1,2,3,4-terahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX) also had a flat dose-response curve against kainate in this model.[17] Regarding the dose-response curves in the AMPA-induced spreading depression test, the curves were usually steeper than in the kainate test, except for **3j** which only partially depressed AMPA response (maximum inhibition: 45% at 2.5 μM).

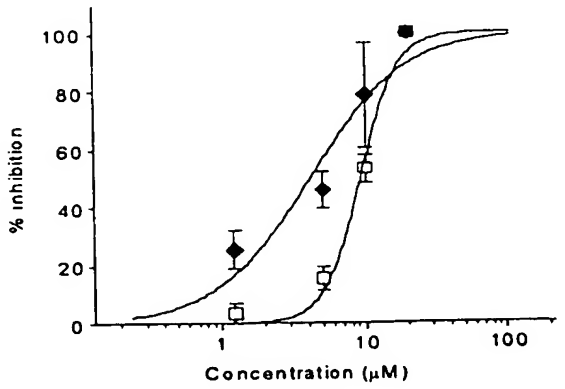


Fig. 2. Inhibition of kainate- and AMPA induced spreading depression in isolated chick retina by **3b**. □ 5 μm AMPA; ◆ 5 μm kainate.

Behavioral changes, effects of the compounds in the maximal electroshock (MES) test, and in the inclined screen test. Most compounds elicited some gross behavioral effects after ip (100 mg/kg) or po (200 mg/kg) application, indicating a reasonable absorption and penetration through the blood brain barrier. These included a decrease in motility, ataxia, muscle weakness and even a loss of righting reflex with the most effective substances. These behavioral symptoms have also been observed with other 2,3-benzodiazepine compounds[5a] and can be regarded as the consequence of the blockade of central AMPA receptors function.

AMPA receptor blockade also results in an anticonvulsant action.[2,5a] MES test has been found a suitable method for establishing in vivo structure–activity relationships among another group of 2,3-benzodiazepine

AMPA antagonists.[5a] The two most effective compounds of the present series were **3b** and **3i**, both with an ED_{50} of 24 mg/kg po, which is between the ED_{50} values of **1** and **2**.

The ataxia inducing or muscle relaxant action was quantified in the inclined screen assay. The ED_{50} values of the compounds in this test (30 min ip pretreatment) were in general slightly higher than in the MES test (60 min oral pretreatment). A bigger gap between the anticonvulsant and motor impairment inducing doses would be expected if identical treatment schedules were used. This MES preference does not hold true in respect of **3a**, which was more potent in the muscle relaxant assay. A possible explanation for this discrepancy is that the compound seems to have an extremely short duration of action (probably due to a rapid metabolism in mice), and 60 min after the treatment its blood concentration decreases to a level that can not protect the animal from MES. Another possible explanation is that—in contrast to most of the 2,3-benzodiazepines[3]—**3a** has a poor absorption from the intestines.

Correlating the in vitro and in vivo pharmacological data with the structural features of the new compounds the following relationships can be established. Applying chloro-, dichloro- or bromo-substitution instead of the methylendioxy group of molecules **1** and **2** and substituting simultaneously the hydrogen bond acceptor type acetyl group of **2** by a condensed nitrogen containing five membered heterocycle preserve the high AMPA antagonist character of the original molecules. Even among the bromo compounds we found a compound (**3k**) with significant activities.

Concerning the condensed heterocycles optimum efficacy was found among the imidazolo[1,2-c][2,3]benzodiazepines of type **3**. Although most of the triazolo-derivatives **4a–d** showed acceptable in vitro efficacy, the additional hydrogen bond acceptor atom in the condensed ring may have prevented a proper absorption resulting in an in vivo inactivity. With pyrrolo-derivative **6**, where no hydrogen bond possibility exists in a similar way as it may occur for molecules **3**, **4** and **2** or where the acceptor nitrogen atom is probably at a wrong place (e.g. imidazolo[3,4-c][3,4]benzodiazepine derivative **5**) neither in vitro nor in vivo effects were found.

Within the imidazolo-derivatives **3** the optimum efficacy was found among the compounds substituted with little aliphatic groups (**3b,c,e,i,k**). Bigger aryl substitutions resulted in inactive or nearly inactive compounds, e.g. **3f,g,h,n**, whereas methyl substitution in position 3 seems to enhance kainate selectivity, e.g. **3c,d,j,l**. But double methyl substitution, e.g. in **3d** and **3m**, resulted in loss of in vivo activity, which may be explained by a worsened ADME profile of these derivatives. It is worth mentioning that **3e**, a homologue of one of the most active substances (**3b**), is inactive in vitro but still has a reasonable anticonvulsant activity. Formation of biologically active metabolites may underlie this discrepancy, which is supported by the observation that the compound was ineffective in the inclined screen assay, where a shorter ip pretreatment was applied. Similar metabolic activation was described among other 2,3-benzodiazepine AMPA antagonists.[5a]

Similarly to earlier 2,3-benzodiazepine type AMPA antagonists[5a,7] the efficacy of the present compounds is also generally restricted to the 4-aminophenyl derivatives (see e.g. the dezamino compounds **3p,q,r**), and even an acetylation of the amino group (e.g. in **3o**) decreases biological activity.

On the basis of the screening results **3b** and in certain cases additionally **3i** were chosen for more detailed and comparative investigations.

Inhibition of AMPA-, or kainate-induced whole-cell currents by 3b in isolated neurons. The AMPA receptor antagonistic effect of **3b** in freshly isolated cerebellar Purkinje cells is shown in Fig. 3. In this preparation **3b** is nearly equiactive with **2** and more potent than **1**. Its IC_{50} against AMPA (2.3 μM) was somewhat lower than against kainate (4.5 μM, not shown). However, under our experimental conditions most current elicited by kainate was probably mediated by AMPA receptors. Thus these data are not suitable for analyzing a possible subtype selectivity of the compound. The current blocking action of **3b** (10 μM) against 5 μM AMPA developed slowly and the recovery from the blockade took several seconds (τ_{on} and τ_{off} of the current trajectories were 4.2±0.8 and 5.2±0.7 sec, respectively), suggesting considerably slower binding and unbinding kinetics than those of **1** and the racemate of **2** (τ_{on} and τ_{off} values below 1 sec).[18] To assess the biological significance of this feature of **3b** warrants further studies. Just as **1** or **2**,[18,19] **3b** acted in a non-competitive way, as its current blocking action was not dependent on the agonist concentration (not shown).

Anticonvulsant profile of selected 2,3-benzodiazepines. The broad-spectrum anticonvulsant activity of **1** and **2** has already been described.[3,5a] Table 4 illustrates that the anticonvulsant profile of **3b** and **3i** is similar to the former compounds. Their potencies were usually between those of **1** and **2**. It is notable, however, that both newly synthesized compounds protected mice from

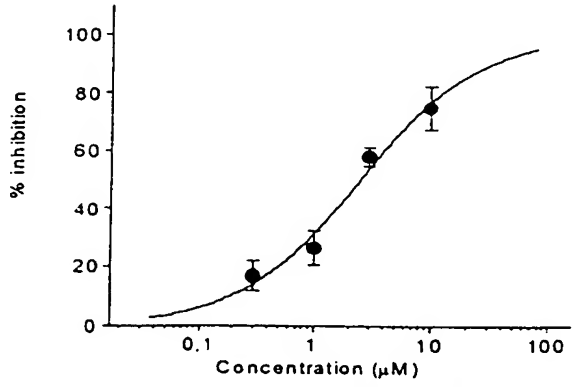


Fig. 3. Inhibition of AMPA induced whole cell current in freshly isolated cerebellar Purkinje cells by **3b**.

Table 4. Anticonvulsive effects of selected compounds (3b and 3i) against MES and various chemical convulsants

	ED_{50} mg/kg po			
	1	2	3b	3i
MES	37.4 (29.2–47.5)	8.6 (7.0–10.6)	24.0 (17.9–32.1)	24.0 (17.9–32.1)
Metrazole	119.8 (108.5–132.3)	16.8 (10.2–27.6)	53.8 (43.7–66.2)	62.2 (44.1–87.6)
Strychnine	86.7 (71.7–104.9)	17.4 (10.6–28.4)	67.4 (43.7–66.2)	44.2 (38.5–50.7)
Bemegride	71.9 (62.0–83.4)	23.9 (16.0–35.7)	40.7(21.9–56.9)	39.9 (26.5–60.1)
Bicuculline	35.0 (28.5–43.1)	14.6 (5.8–36.7)	22.8 (16.7–31.2)	22.5 (13.4–37.7)
Nicotine	71.8 (49.8–103.7)	22.7 (16.2–31.9)	13.1 (8.1–21.2)	18.3 (14.2–23.8)
4-aminopyridine	43.0 (39.9–59.8)	8.4 (5.6–12.5)	55.5 (47.1–65.4)	6.7 (3.5–12.7)
3-mercapto-propionic acid	47.0 (32.9–67.2)	17.1 (9.7–30.1)	38.1 (28.7–50.5)	66.8 (43.0–103.1)

nicotine-induced seizures and death more potently than 2 did, which may indicate a potential antiparkinsonian efficacy. Further, 3i was the most effective of the four compounds in preventing 4-aminopiridine (4-AP)-induced seizures.

Muscle relaxant effect of selected 2,3-benzodiazepines. A common feature among 2,3-benzodiazepine AMPA antagonists is the inhibition of motor functions with a primarily spinal site of action.[3,20] The ataxia/muscle relaxation inducing ED_{50} values of **3b** and **3i** are listed and compared to those of **1** and **2** in the inclined screen and rotarod tests (Table 5).

Antiischemic effect of 3b in a transient focal ischemia model, in rats. The antiischemic property of AMPA antagonists renders them promising drug candidates in various acute and chronic neurodegenerative disorders such as stroke.[1–3] Compound **1** has been shown to protect animals from ischemic damage in various models.[21] The transient focal ischemia model is a relevant stroke model.[22,23] Table 6 shows that **3b**, applied at 2.5–5-fold lower doses, had a considerably more potent effect on infarct size than **1**.

The antiparkinsonian effects of 3b

Oxotremorine induced tremor. The cholinergic agonist oxotremorine, applied systemically, provokes tremor with a central action. Table 7 shows that the 2,3-benzodiazepine compounds **1**, **2** and **3b** potently mitigated the tremor induced by oxotremorine. Salivation, a peripheral effect of oxotremorine, was not affected by these drugs. Since Parkinson's disease is characterized by a disturbed balance between cholinergic and dopaminergic neurotransmission in the basal ganglia, an indirect central anticholinergic effect of compounds **1**, **2** and **3b** may be indicative for a therapeutically suitable antiparkinsonian effect.

Table 6. Effect of 3b on the size of ischemic damage in rats after transient occlusion of the medial cerebral artery

Comp.	Doses (mg/kg iv)	Infarct size scores (mean ± S.E.)	Decrease (%)
Veh.[a]		48920±4012	
1	6×5	32218±6325*	34.1
Veh.[a]		40518±5928	
3b	6×2	13229±2313*	67.4
Veh.[a]		42795±7513	
3b	6×1	16122±4368*	62.3

[a]Vehicle. * $P<0.01$ Significance was calculated by one-way ANOVA followed by the Duncan test. Treatment schedule: first injection 30 min after occlusion and repeated 5 times in every 30 min.

Table 7. Antagonism of oxotremorine induced tremor in mice

Oxotremorine antagonism ED_{50} mg/kg po		
1	2	3b
20.5 (14.9–28.3)	5.6 (3.6–8.5)	16.8 (12.0–23.6)

***N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine·HCl (MPTP) neurotoxicity.** Degeneration of dopaminergic neurons in Parkinsons disease is well known.[24] Recent findings indicate that impairment of striatal dopaminergic neurotransmission results in an overactivity of glutamatergic neurons in the subthalamic nucleus.[25] This glutamatergic overactivity is thought to play an important role in the expression of some parkinsonian symptoms, such as hypokinesia and rigidity as well as in development of neuronal damage.[26] Therefore we tested the effects of **3b** against MPTP-induced neurotoxicity in mice, a relevant rodent model of Parkinsons disease, in comparison with those of **1** and of the competitive AMPA antagonist NBQX.

Table 5. Muscle relaxant effects of selected compounds (3b and 3i) in the inclined screen and rotarod tests, in mice

	ED_{50} mg, kg ip			
Method	1	2	3b	3i
Inclined screen	47.1 (44.2–50.2)	13.4 (11.2–16.0)	36.5 (29.4–45.2)	47.2 (41.9–53.3)
Rotarod	24.0 (22.0–26.2)	2.3 (1.6–3.4)	15.8 (13.0–19.3)	13.7 (12.4–15.1)

Table 8. Effects of 3b on MPTP induced depletion of dopamine and its metabolites in mouse striatum[a]

Treatment					Concentration (μg/g) of		
1 0 min.	2 30 min.	3 150 min.	4 270 min.	5 390 min.	DA	DOPAC	HVA
Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	11.14±0.43	0.54±0.03	0.87±0.05
MPTP	Vehicle	Vehicle	Vehicle	Vehicle	1.07±0.17[b]	0.10±0.04[b]	0.33±0.06[b]
MPTP	3b	3b	Vehicle	3b	4.38±0.89[c]	0.21±0.05	0.45±0.06
MPTP	1	1	1	Vehicle	3.60±0.37[c]	0.27±0.03[d]	0.36±0.03
MPTP	NBQX	NBQX	NBQX	Vehicle	2.74±0.52[d]	0.16±0.04	0.37±0.03

[a]Significance was calculated by one-way ANOVA followed by the Duncan's test.
[b] < 0.01 versus saline treated control group.
[c] $P < 0.01$ versus MPTP treated group.
[d] $P < 0.05$ versus MPTP treated group.

Table 9. Determination of acute toxicity in mice

Compound	LD_{50} mg/kg	
	ip	po
1	302.1 (273.9–333.3)	296.9 (258.6–340.8)
3b	259.4 (205.8–327.1)	372.9 (322.5–431.1)

The effects of 3×20 mg/kg (ip dose) of **3b**, **1** and NBQX on MPTP-induced dopamine (DA) depletion in mouse striatum are shown in Table 8. MPTP injection reduced DA concentration in the striatum by 90% and the levels of DA metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid, (HVA) were also decreased significantly (Table 8). MPTP administration did not affect the concentrations of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) in the striatum indicating the specific toxic effect of MPTP on dopaminergic neurons (data not shown). **3b** and **1**, when they were injected repeatedly, reversed the MPTP-induced decrease in striatal concentrations and NBQX exhibited similar effect (Table 8). The neuroprotective effects of 2,3-benzodiazepines against MPTP neurotoxicity suggest that non-competitive AMPA receptor antagonists may have a beneficial role in the treatment of Parkinson's disease.[25,26]

Acute toxicity. Compound **3b** caused lethality in mice only at doses several fold higher than its ED_{50} values in the pharmacological assays. Its toxicity is similar to that of **1**. The LD_{50} values of **3b** and **1** were almost the same after ip and oral administrations suggesting a good gastro-intestinal absorption (Table 9).

Conclusion

Among the newly synthesized, with heterocycle condensed and halogen substituted 2,3-benzodiazepine derivatives several non-competitive AMPA antagonists were identified with potencies that approached or exceeded those of the reference compounds **1** or **2**. Some of them also had potent in vivo activity. **3b**, the compound selected for detailed studies, displayed a pharmacological profile largely similar to that of the formerly described 2,3-benzodiazepine AMPA antagonists.[3] but a potent anti-parkinsonian activity was also revealed as an additional feature. The compound showed an excellent, broad spectrum anticonvulsant activity against seizures evoked by electroshock and different chemoconvulsive agents indicating a possible antiepileptic efficacy. **3b** (GYKI 47261) was also found to be highly active in a transient model of focal ischemia predictive of a therapeutic value in human stroke. A significant effect antagonizing the MCAO induced infarct size in rat brain was found in a five times lower dose than for GYKI 52466 (**1**), the representative of the non competitive AMPA antagonists. In addition **3b** reversed the dopamine depleting effect of MPTP and antagonized the oxotremorine induced tremor in mice. These findings indicate that the non-competitive AMPA antagonist **3b** may have therapeutical potential not only in acute but chronic neurodegenerative disorders as well.[27]

Experimental

Chemistry

Melting points were measured on a Boëtius hotstage microscope and are uncorrected. IR (KBr): Bruker IFS-85 spectrophotometer. ^{1}HNMR ($CDCl_3$, internal standard TMS, T = 298°K): Bruker AC 250, other solvents are indicated. Mass spectra: Finnigan MAT 8430 mass spectrometer. Operating conditions: electron ionization, $E_{el} = 70$eV, $I_{el} = 0.5$ mA, $U_{acc} = 3$ kV, R = 1250. FAB: ION-TECH atom gun with Xe, in *m*-nitrobenzyl alcohol matrix. Column chromatography: silica gel, Kieselgel 60, Merck. Satisfactory elemental analyses (±0.4 %) for *C*, *H*, *N* and *S* were obtained for all new described compounds except for **25–29** which were not measured this way. The following compounds were prepared according to the literature: **7a**,[28] **7b**,[28] **7c**,[29] **13a**,[30] **13b**,[31] **13c**,[30] **13d**,[30] **13e**,[31] **13f**,[32] **13g**.[32]

General procedure for the synthesis of isochromanes 9a–d. To a stirred solution of the phenylethanol derivative (**7a–c**) (0.1 mol) and the corresponding benzaldehyde **8a,b** (0.1 mol) in dry benzene (300 mL) freshly molten and ground zinc chloride (13.6 g, 0.1 mol) was given. Dry hydrogen chloride gas was introduced into the suspension over 4 h and the mixture was stirred overnight. The resulting mixture was then washed with water and a 5% sodium-hydrogensulfit solution. The organic phase was dried and after filtration the solvent evaporated. The residue was recrystallized to give the title products.

7-Chloro-1-(4-nitrophenyl)isochromane (9a). Yield: 56%. Mp 98–101 °C (ethanol). $C_{15}H_{12}ClNO_3$ (289.7). ^{1}H NMR: δ 2.79 (ddd, J_1 = 16.5 Hz, $J_2 = J_3$ = 3.6 Hz, 4-H) and 3.12 (ddd, J_1 = 16.5 Hz, J_2 = 9.7 Hz, J_3 = 5.6 Hz, 4-H), 3.92 (ddd, J_1 = 11.5 Hz, J_2 = 9.7 Hz, J_3 = 3.6 Hz, 3-H) and 4.21 (ddd, J_1 = 16.5 Hz, J_2 = 5.6 Hz, J_3 = 3.6 Hz, 3-H), 5.75 (s, 1H, 1-H), 6.65 (d, J = 1.9 Hz, 1H, 8-H), 7.13 (d, J = 8.3 Hz, 1H, 5-H), 7.17 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H, 6-H), 7.50 (d, J = 8.8 Hz, 2H) and 8.25 (d, J = 8.8 Hz, 2H, nitrophenyl).

7-Bromo-1-(4-nitrophenyl)isochromane (9b). Yield: 62%. Mp 104–107 °C (ethyl acetate). $C_{15}H_{12}BrNO_3$ (334.2). ^{1}H NMR: δ 2.78 (ddd, J_1 = 16.2 Hz, $J_2 = J_3$ = 3.6 Hz, 4-H) and 3.12 (ddd, J_1 = 16.2 Hz, J_2 = 9.9 Hz, J_3 = 5.8 Hz, 4-H), 3.93 (ddd, J_1 = 13.5 Hz, J_2 = 9.9 Hz, J_3 = 3.6 Hz, 3-H) and 4.21 (ddd, J_1 = 13.5 Hz, J_2 = 5.8 Hz, J_3 = 3.6 Hz, 3-H), 5.78 (s, 1-H), 6.82 (d, J = 1.9 Hz, 8-H), 7.10 (d, J = 8.2 Hz, 5-H), 7.35 (dd, J_1 = 8.2 Hz, J_2 = 1.9 Hz, 6-H), 7.55 (d, J = 8.8 Hz, 2H) and 8.22 (d, J = 8.8 Hz, 2H, nitrophenyl).

7,8-Dichloro-1-(4-nitrophenyl)isochromane (9c). Yield: 30%. Mp 130–132 °C (ethanol). $C_{15}H_{11}Cl_2NO_3$ (324.1). ^{1}H NMR: δ 2.80 (ddd, J_1 = 16.9 Hz, $J_2 = J_3$ = 3.9 Hz, 4-H) and 3.13 (ddd, J_1 = 16.9 Hz, J_2 = 10.0 Hz; J_3 = 5.9 Hz, 4-H), 3.91 (ddd, J_1 = 11.5 Hz, J_2 = 10.0 Hz, J_3 = 3.9 Hz, 3-H) and 4.20 (ddd, J_1 = 11.5 Hz, J_2 = 5.9 Hz, J_3 = 3.9 Hz, 3-H), 5.72 (s, 1H, 1-H), 6.76 (s, 1H, 8-H), 7.30 (s, 1H, 5-H), 7.48 (d, J = 8.7 Hz, 1H) and 8.23 (d, J = 8.7 Hz, 1H, nitrophenyl).

7-Bromo-1-(2-chlorophenyl)isochromane (9d). Yield: 40%. Mp 62–65 °C (ethanol). $C_{15}H_{12}BrClO$ (323.6). ^{1}H NMR: δ 2.75 (ddd, J_1 = 16.4 Hz, $J_2 = J_3$ = 3.6 Hz, 4-H) and 3.09 (ddd, J_1 = 16.4 Hz, J_2 = 9.7 Hz, J_3 = 5.7 Hz, 4-H), 3.93 (ddd, J_1 = 13.6 Hz, J_2 = 9.7 Hz, J_3 = 3.6 Hz, 3-H) and 4.21 (ddd, J_1 = 13.6 Hz, J_2 = 5.7 Hz, J_3 = 3.6 Hz, 3-H), 6.88 (d, J = 1.7 Hz, 1H, 8-H), 7.05 (d, J = 8.0 Hz, 1H, 5-H), 7.14–7.34 (m, 4H, 2-clorophenyl), 7.45 (dd, J_1 = 8.0 Hz, J_2 = 1.7 Hz, 1H, 5-H).

General procedure for the synthesis of phenylacetic acid derivatives 10a–d. To a solution of the corresponding isochromane (**9a–d**) (90 mmol) in acetone (360 mL) Jones reagent (260 mL) was gradually given and the mixture was stirred for 16 h. The separated chromium sulfate was filtered and the filtrate evaporated to dryness. The residue was treated with excess 10% sodium carbonate solution and dichloromethane. After separation the aqueous layer was acidified with concd hydrochloric acid and the separated crystals were collected by filtration.

4-Chloro-2-(4-nitrobenzoyl)phenylacetic acid (10a). Yield: 78%. Mp 150–152 °C (ethyl acetate). $C_{15}H_{10}ClNO_5$ (319.7). ^{1}H NMR: (DMSO-d_6): δ 3.82 (s, 2H, $C\underline{H}_2$), 7.42 (d, J = 1.8 Hz, 1H, 3-H), 7.48 (d, J = 8.2 Hz, 1H, 6-H), 7.62 (dd, J_1 = 8.2 Hz, J_2 = 1.8 Hz, 1H, 5-H), 7.90 (d, J = 8.7 Hz, 2H) and 8.32 (d, J = 8.7 Hz, 2H, nitrobenzoyl).

4-Bromo-2-(4-nitrobenzoyl)phenylacetic acid (10b). Yield: 62 %. Mp 128–130 °C. $C_{15}H_{10}BrNO_5$ (364.2). ^{1}H NMR: (DMSO-d_6): δ 3.80 (s, 2H, $C\underline{H}_2$), 7.46 (d, J = 8.2 Hz, 1H, 6-H), 7.57 (d, J = 2.0 Hz, 1H, 3-H), 7.81 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 5-H), 7.95 (d, J = 8.9 Hz, 2H) and 8.38 (d, J = 8.9 Hz, 2H, nitrobenzoyl), 12.1–12.7 (1H).

4,5-Dichloro-2-(4-nitrobenzoyl)phenylacetic acid (10c). The crude product prepared by the evaporation of its solution in acetone was recrystallized from 96% acetic acid and further purified by column chromatography. Eluent: chloroform:methanol (9:1). Yield: 54 %. Mp 187–190 °C. $C_{15}H_9Cl_2NO_5$ (354.1). ^{1}H NMR: (DMSO-d_6): δ 3.90 (s, 2H, $C\underline{H}_2$), 7.68 (s, 1H), 7.85 (s, 1H), 7.97 (d, J = 8.7 Hz, 2H) and 8.38 (d, J = 8.7 Hz, 2H, nitrobenzoyl).

4-Bromo-2-(2-chlorobenzoyl)phenylacetic acid (10d). Yield: 47%. Mp 135–138 °C. $C_{15}H_{10}BrClO_3$ (353.6). ^{1}H NMR: (DMSO-d_6): 3.90 (s, 2H, $C\underline{H}_2$), 7.37 (d, J = 2.2 Hz, 1H, 3-H), 7.43 (d, J = 8.1 Hz, 1H, 6-H), 7.78 (dd, J_1 = 8.1 Hz, J_2 = 2.2 Hz, 1H, 5-H), 7.47 (m, 2H) and 7.60 (m, 2H, chlorophenyl), 12.40 (br. s).

8-Chloro-1-(4-nitrophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepin-4-one (11a). Method A. A solution of **10a** (17.6 g, 55.0 mmol) and 85% hydrazine hydrate (8 mL) in ethanol (340 mL) was heated at reflux for 4 h. The mixture was then chilled, treated with 1N hydrochloric acid (115 mL) and the solvent was evaporated. The residue was treated with water (50 mL) and the precipitate filtered and dried. This intermediate hydrazone was dissolved in dichloromethane (300 mL) and treated with a solution of dicyclohexylcarbodiimide (13.4 g 65.0 mmol) in dichloromethane (210 mL). The mixture was stirred overnight at rt and the resulting precipitate was filtered and washed with dichloromethane to give **11a** (12.5 g). Yield: 72 %. Mp 275–278 °C.

Method B. The reaction was performed as in Method A, but isopropanol was used as solvent and after reflux it was treated with 1N hydrochloric acid (125 mL) and stirred overnight at rt The resulting precipitate was filtered and the crude product was recrystallized from 2-methoxyethanol. Yield: 88%. Mp 275–277 °C. $C_{15}H_{10}ClN_3O_3$ (315.7). ^{1}H NMR: (DMSO-d_6): δ 3.62 (s, 2H, 5-H), 7.20 (d, J = 2.0 Hz, 1H, 9-H), 7.58 (d, J = 8.2 Hz, 1H, 6-H), 7.70 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 7-H), 7.80 (d, J = 8.7 Hz, 2H) and 8.30 (d, J = 8.7 Hz, 2H, nitrophenyl).

8-Bromo-1-(4-nitrophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepin-4-one (11b). Starting from **10b** (12.7 g, 35.0 mmol) Method A was essentially followed as described for **11a**. Yield: 65% (8.19 g). Mp 264–267 °C. $C_{15}H_{10}BrN_3O_3$ (360.2). ^{1}H NMR: (DMSO-d_6): δ 3.60 (br. s, 2H, 5-H), 7.29 (d, J = 1.8 Hz, 1H, 9-H), 7.52 (d, J = 8.3 Hz, 1H, 6-H), 7.77 (overlapping, 7-H), 7.78 (d, J = 8.7 Hz) and 8.32 (d, J = 8.7 Hz, 2H, nitrophenyl), 11.41 (s, 1H, N-$\underline{H}$).

7,8-Dichloro-1-(4-nitrophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepin-4-one (11c). A solution of **10c** (6.1 g, 17.2 mmol) and 85% hydrazine hydrate (6 mL) in isopropanol (300 mL) was treated at reflux for 6 h. The solvent was evaporated and the residue was dissolved in a mixture of 40% acetic acid (40 mL) and dichloromethane (400 mL). After separation the organic layer was washed with water, dried and treated with dicyclohexylcarbodiimide (3.60 g 17.5 mmol). The reaction mixture was stirred

overnight and the precipitate filtered. The filtrate was evaporated to dryness and the residue was boiled with methanol (120 mL) and filtered while hot to give the product (4.40 g). Yield: 73%. Mp 268–270 °C. $C_{15}H_9Cl_2N_3O_3$ (350.1). ^{1}H NMR: (DMSO-d_6): δ 3.76 (s, 2H, 5-H), 7.48 (s, 1H, 6-H), 7.90 (d, J = 8.8 Hz, 2H) and 8.39 (d, J = 8.8 Hz, 2H, nitrophenyl), 8.01 (s, 1H, 9-H).

8-Bromo-1-(2-chlorophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepin-4-one (11d). A solution of **10d** (4.28 g, 12.1 mmol) and 98% hydrazine hydrate (1.8 mL) in ethanol (45 mL) was refluxed for 5 h. The mixture was evaporated to dryness and the residue was dissolved in dichhorometane (120 mL) and washed with water (3×20 mL). The organic layer was dried and evaporated. The crude product was recystallized from ethanol to give the product (2.26 g). Yield: 53%. Mp 255–258 °C. $C_{15}H_{10}BrClN_2O$ (349.6). ^{1}H NMR: (DMSO-d_6): δ 3.62 (s, 2H, 5-H), 7.02 (d, J = 1.8 Hz, 1H, 9-H), 7.52 (d, J = 8.1 Hz, 1H, 6-H), 7.79 (dd, J_1 = 8.1 Hz, J_2 = 1.8 Hz, 1H, 7-H), 7.55–7.75 (m, 4H, 2-chlorophenyl), 11.35 (br. s, 1H, 3-H).

General method for the synthesis of 3,5-dihydro-4*H*-2,3-benzodiazepine-4-thione derivatives 12a–d. To a solution of the corresponding 4-oxo-2,3-benzodiazepine derivative (**11a–d**) (38.0 mmol) in dry pyridine (150 mL) phosphorous pentasulfide (13.3 g, 60.0 mmol) was given and the mixture was kept at 80 °C for 2–3 h. The reaction mixture was then cooled to rt and poured onto ice (1 kg). The precipitate was collected by filtration and washed with water. The crude products were recrystallized from 2-methoxyethanol.

8-Chloro-1-(4-nitrophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thione (12a). Yield: 71%. Mp 231–234 °C. $C_{15}H_{10}ClN_3O_2S$ (331.8). ^{1}H NMR: (DMSO-d_6): δ 4.00 (br. s, 2H, 5-H), 7.48 (d, J = 8.2 Hz, 1H, 6-H), 7.15 (d, J = 1.9 Hz, 1H, 9-H), 7.72 (dd, J_1 = 8.2 Hz, J_2 = 1.9 Hz, 1H, 7-H), 7.80 (d, J = 8.7 Hz, 2H) and 8.28 (d, J = 8.7 Hz, 2H, nitrophenyl).

8-Bromo-1-(4-nitrophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thione (12b). Yield: 67%. Mp 220–223 °C. $C_{15}H_{10}BrN_3O_2S$ (376.8). ^{1}H NMR: (DMSO-d_6): δ 4.05 (br. s, 2H, 5-H), 7.35 (d, J = 1.8 Hz, 1H, 9-H), 7.48 (d, J = 8.3 Hz, 1H, 6-H), 7.88 (dd, J_1 = 8.3 Hz, J_2 = 1.8 Hz, 1H, 7-H), 7.84 (d, J = 8.8 Hz, 2H) and 8.36 (d, J = 8.8 Hz, 2H, nitrophenyl), 13.1 (s, 1H, N-H).

7,8-Dichloro-1-(4-nitrophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thione (12c). Yield: 61%. Mp 210–213 °C. $C_{15}H_9Cl_2N_3O_2S$ (366.2). MS(EI): *m/z*: M: 366 368.

8-Bromo-1-(2-chlorophenyl)-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thione (12d). Yield: 79%. Mp 198–20 °C. $C_{15}H_{10}BrClN_2S$ (365.7). MS(EI): *m/z*: M: 365/367.

General procedure for the synthesis of 11*H*-imidazolo[1,2-c][2,3]-benzodiazepine derivatives (15a–g, 15i–n, 3p, q). A mixture of the appropriate 2,3-benzodiazepine-4-thione derivative (**12a–d**) (10.0 mmol), the corresponding aminoacetal (**13a–g**) (20.0 mmol) and red mercury oxide (10.0 mmol) in 2-methoxyethanol was stirred and heated at reflux for 1–10 h. After filtration the solvent was removed and the residue was purified by chromatography, eluent was chloroform:methanol (98:2). The fractions containing the condensation product of type 14 were evaporated to dryness and the residue was treated with a 1:1 mixture of coned hydrochloric acid and ethanol and heated at reflux for 1–2 h. Evaporation gave the title products as hydrochloride salts. In a few cases (15f,g,n) the intermediate condensation product was reacted with methanesulfonic acid at rt for 1–2 h to induce the ring closure reaction. In these cases the reaction mixture was diluted with water and made alkaline with 5N sodium hydroxide. The products were collected by filtration. The yields, physical and spectroscopic date are collected in Table 10.

8-Chloro-1-(4-nitrophenyl)-3-(2-oxopropyl)-3,5-dihydro-4*H*-2,3-benzodiazepin-4-one (16). To a solution of **11a** (36.3 g, 115 mmol) in DMF (250 mL) potassium carbonate (19.9 g, 144 mmol) was given and the mixture was heated at 80 °C for 10 min. Then chloroacetone (11 mL, 138 mmol) was added and the mixture was heated at 80 °C for 2 h. The reaction mixture was then cooled to rt and poured onto ice (1.2 kg). The precipitate was collected by filtration and washed with water. The crude product was recrystallized from acetic acid to give **16** (39.3 g). Yield: 92%. Mp 222–224 °C. $C_{18}H_{14}ClN_3O_4$ (371.8). ^{1}H NMR: (DMSO-d_6): δ 2.12 (s, 3H, CH_3), 3.75 (br. s, 2H, 5-H), 4.72 (s, 2H, CH_2), 7.23 (d, J = 2.1 Hz, 1H, 9-H), 7.63 (d, J = 8.3 Hz, 1H, 6-H), 7.74 (dd, J_1 = 8.3 Hz, J_2 = 2.1 Hz, 1H, 7-H), 7.83 (d, J = 8.8 Hz, 2H) and 8.33 (d, J = 8.8 Hz, 2H, nitrophenyl).

8-Chloro-1-(4-nitrophenyl)-3-phenacyl-3,5-dihydro-4*H*-2,3-benzodiazepin-4-one (17). The compound was synthesized from **11a** according to the procedure as given above by using 2-chloroacetophenone. Yield: 83%. Mp 223–225 °C. $C_{23}H_{16}ClN_3O_4$ (433.8). ^{1}H NMR: (DMSO-d_6): δ 3.8 (br. s, 2H, 5-H), 5.4 (br. s, 2H, phenacyl), 7.22 (d, J = 1.9 Hz, 1H, 9-H), 7.64 (d, J = 8.3 Hz, 1H, 6-H), 7.74 (dd, J_1 = 8,3 Hz, J_2 = 1.9 Hz, 1H, 7-H), 7.54 (dd, J_1 = J_2 = 7.5 Hz, 2H, phenyl), 7.66 (dd, J_1 = J_2 = 7.5 Hz, 1H, phenyl), 7.98 (d, J = 7.5 Hz, 2H, phenyl), 7.83 (d, J = 8.8 Hz, 2H) and 8.32 (d, J = 8.8 Hz, 2H, nitrophenyl).

8-Chloro-2-methyl-6-(4-nitrophenyl)-11*H*-imidazolo[1,2-c][2,3]benzodiazepine hydrochloride (15b). (Alternative route.) To a solution of ammonium acetate (180 g) in acetic acid (180 mL) **16** (9.0 g, 24.2 mmol) was added and the mixture was heated at 140 °C for 3 h. The reaction mixture was then cooled to rt and poured onto ice (0.9 kg). The precipitate was collected by filtration and washed with water. The crude product was recrystallized twice from DMF to give the product as base (3.42 g). Yield: 40%. Mp 246–250 °C. A sample was converted to the hydrochloride with mp 230–231 °C which was identical with the substance prepared by the general method described earlier, for further data see Table 10.

8-Chloro-6-(4-nitrophenyl)-2-phenyl-11*H*-imidazolo[1,2-c][2,3]benzodiazepine (15h). The compound was prepared from **17** according to the procedure as described above for **15b**. (For yield, physical and spectroscopic data see Table 10.)

Table 10. Physical and spectroscopic data of the 11*H*-imidazolo[1,2-c][2,3]benzodiazepine derivatives 15a n, 3p q

Compound[a]	mp (°C)	Yield (%)	Molecular formula (Mol. mass)	Spectroscopic data: ^{1}H NMR (in DMSO-d_6; δ) or MS
15 a	210–215	48	$C_{17}H_{12}Cl_2N_4O_2$ (375.2)	4.55 (s, 2H, 11-H), 7.22 (d, J = 1.8 Hz, 1H, 7-H), 7.65 (d, J = 8.2 Hz, 1H, 10-H), 7.84 (dd, J_1 = 8.2 Hz, J_2 = 1.8 Hz, 1H, 9-H), 7.62 (overlapping) and 7.95 (2-H and 3-H), 8.00 (d, J = 8.5 Hz, 2H) and 8.43 (d, J = 8.5 Hz, 2H, nitrophenyl).
15 b	229–230	79	$C_{18}H_{14}Cl_2N_4O_2$(389.2)	2.25 (d, J = 1.0 Hz, 3H, 2-CH_3), 4.52 (s, 2H, 11-H), 7.23 (d, J = 1.8 Hz, 1H, 7-H), 7.63 (d, J = 8.2 Hz, 1H, 10-H), 7.72 (q, J = 1.0 Hz, 1H, 3-H), 7.82 (dd, J_1 = 8.2 Hz, J_2 = 1.8 Hz, 1H, 9-H), 7.96 (d, J = 8.6 Hz) and 8.44 d, J = 8.6 Hz, nitrophenyl).
15 c	205–208	41	$C_{18}H_{14}Cl_2N_4O_2$(389.2)	2.40 (d, J = 1.0 Hz 3H, 3-CH_3), 7.22 (d, J = 1. Hz, 1H, 7-H), 7.42 (q, J = 1.0 Hz, 1H, 2-H), 7.62 (d, J = 8.2 Hz, 1H, 10-H), 7.86 (dd, J_1 = 8.2 Hz, J_2 = 1.8 Hz, 1H, 9-H), 8.05 (d, J = 8.6 Hz) and 8.40 /d, J = 8.6 Hz, nitrophenyl).
15 d	207–210	46	$C_{19}H_{16}Cl_2N_4O_2$(403.3)	2.22 (s, 3H), 2.35 (s, 3H), 4.49 (s, 2H, 11-H), 7.22 (d, J = 1.9 Hz, 1H, 7-H), 7.63 (d, J = 8.2 Hz, 1H, 10-H), 7.86 (dd, J_1 = 8.2 Hz, J_2 = 1.9 Hz, 1H, 9-H), 8.02 (d, J = 8.7 Hz) and 8.40 (d, J = 8.7 Hz, nitrophenyl).
15 e	150–153	20	$C_{19}H_{16}Cl_2N_4O_2$(403.3)	MS(EI): m/z: M: 403/405.
15 f[b]	270–272	30	$C_{23}H_{14}ClN_5O_4$(459.8)	4.25 (br. s, 2H, 11-H), 7.26 (d, J = 1.8 Hz, 1H, 7-H), 7.55 (s, 1H, 2-H), 7.72 (d, J = 8.3 Hz, 1H, 10-H), 7.76 (dd, J_1 = 8.3 Hz, J_2 = 1.8 Hz, 1H, 9-H), 7.95 (d, J = 8.9 Hz, 2H), 8.04 (d, J = 8.9 Hz, 2H) and 8.36 (d, J = 8.9 Hz, 2H), 8.41 (d, J = 8.9 Hz, nitrophenyls).
15 g[b]	250–252	30	$C_{22}H_{14}ClN_5O$(415.8)	4.10 (br. s, 2H, 11-H), 7.22 (d, J = 1.9 Hz, 1H, 7-H), 7.35 (s, 1H, 2-H), 7.47 (d, J = 8.3 Hz, 1H, 10-H), 7.56 (d, J = 6.2 Hz, 2H, pyridyl), 7.60 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H, 9-H), 7.92 (d, J = 8.9 Hz, 2H) and 8.37 (d, J = 8.9 Hz, 2H, nitrophenyl), 8.72 (d, J = 6.2 Hz, 2H, pyridyl).
15 h[b]	239–232	82	$C_{23}H_{15}ClN_4O_2$414.8	4.25 (br. s, 2H, 11-H), 7.21 (br. s, 1H, 7-H), 7.72 (br. s, 2H, 9-H and 10-H), 7.12–7.26 (1H, phenyl, overlapping), 7.37 (dd, J_1 = l_2 = 7.3 Hz, 2H, phenyl), 7.78 (d, J = 7.3 Hz, 2H, phenyl), 8.00 (d, J = 8.8 Hz, 2H, nitrophenyl), 8.10 (s, 1H, 3-H), 8.42 (d, J = 8.8 Hz, nitrophenyl).
15 i	221–224	13	$C_{18}H_{13}Cl_3N_4O_2$(423.7)	MS (EI): *m/z* (%): M 386/388 (100/68), 385/387 (45/30), 339/341 (16/12).
15 j	240–244	41	$C_{18}H_{13}Cl_3N_4O_2$(423.7)	MS (EI): *m/z* (%): M 386/388 (73/45), 385/387 (100/68), 339/341 (20/14).
15 k	215–220	28	$C_{18}H_{14}BrClN_4O_2$(433.8)	2.25 (d, J = 1.0 Hz, 3H, 2-CH_3), 4.43 (br. s, 2H, 11-H), 7.35 (d, J = 1.9 Hz, 1H, 7-H), 7.58 (d, J = 8.3 Hz, 1H, 10-H), 7.71 (q, J = 1.0 Hz, 1H, 3-H), 7.96 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H, 9-H), 7.98 (d, J = 8.82 Hz) and 8.43 (d, J = 8.8 Hz, nitrophenyl).
15 l	194–202	50	$C_{18}H_{14}BrClN_4O_2$(433.8)	2.42 (d, J = 1.0 Hz, 3H, 3-CH_3), 4.50 (s, 2H, 11-H), 7.32 (d, J = 1.8 Hz, 1H, 7-H), 7.42 (q, J = 1.0 Hz, 1H, 2-H), 7.55 (d, J = 8.1 Hz, 1H, 10-H), 7.97 (dd, J_1 = 8.1 Hz, J_2 = 1.8 Hz, 1H, 9-H), 8.05 (d, J = 8.7 Hz) and 8.42 (d, J = 8.7 Hz, nitrophenyl).
15 m	212–219	42	$C_{19}H_{16}BrClN_4O_2$(447.8)	2.22 (s, 3H), 2.34 (s, 3H), 4.45 (s, 2H, 11-H), 7.42 (d, J = 1.9 Hz, 1H, 7-H), 7.58 (d, J = 8.1 Hz, 1H, 10-H), 7.95 (dd, J_1 = 8.1 Hz, J_2 = 1.9 Hz, 1H, 9-H), 8.01 (d, J = 8.8 Hz) and 8.42 (d, J = 8.8 Hz, nitrophenyl).
15 n[b]	Foam	60	$C_{22}H_{14}BrN_5O$(460.3)	MS(EI): m/z: M: 460/462.
3 p[c,d]	—	—	$C_{18}H_{14}BrCl_2N_3$(423.2)	
3 q[c,d]	—	—	$C_{18}H_{14}BrCl_2N_3$(423.2)	

[a]Hydrochlorides.
[b]Data refer to the base.
[c]For Mp and yield see Table 1.
[d]For spectroscopic data see Table 12.

8-Chloro-4-methylthio-1-(4-nitrophenyl)-5*H*-2,3-benzodiazepine (18). Thione **12a** (3.32 g 10.0 mmol) was dissolved in acetone (200 mL) and potassium carbonate (2.76 g 20.0 mmol) and methyliodide (1.87 mL, 30.0 mmol) were added. The mixture was stirred at rt for 3 h, then the product was filtered, washed with water and recrystallized from DMF to give the title product (2.84 g). Yield: 82%. Mp 249–252 °C. $C_{16}H_{12}ClN_3O_2S$ (345.8). ^{1}H NMR: δ 2.42 (s, 3H, S-CH_3), 3.35 (d, J = 13.0 Hz, 5-H), and 3.45 (d, J = 13.0 Hz), 7.20 (d, J = 2.0 Hz, 1H, 9-H), 7.25 (d, J = 8.2 Hz, 1H, 6-H), 7.55 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 7-H), 7.87 (d, J = 8.8 Hz) and 8.38 (d, J = 8.8 Hz, nitrophenyl).

General procedure for the synthesis of 3-substitued 8-chloro-6-(4-nitrophenyl)-11*H*-1,2,4-triazolo[4,5-c][2,3]benzodiazepine derivatives (19a–d). 18 (3.45 g 10.0 mmol) was dissolved in DMF (120 mL) then the corresponding acylhydrazide (25.0 mmol) and concd HCl (0.5 mL) were added and the mixture was stirred and heated at 120–130 °C for 9–15 h. The reaction mixture was poured onto ice and the product filtered. The crude products were recrystallized. The yields, physical and analytical data are collected in Table 11.

7-Chloro-3-methyl-1-(4-nitrophenyl)isochromane (21). Prepared from 1-(4-chlorophenyl)-2-propanol[33] according to the general method as described for **9a–d**. Yield: 32%. Mp 120–123 °C. $C_{16}H_{14}ClNO_3$ (303.8). ^{1}H NMR: δ 1.42 (d, J = 6.4 Hz, 3H, 3-CH_3), 2.7–2.95 (m, 2H, 4-H), 4.02 (m, 1H, 3-H), 5.80 (s, 1H, 1-H), 6.60 (d, J = 2.0 Hz, 1H, 8-H), 7.10 (d, J = 8.2 Hz, 1H, 5-H), 7.16 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 6-H), 7.53 (d, J = 8.8 Hz, 2H) and 8.24 (d, J = 8.8 Hz, 2H, nitrophenyl).

7-Chloro-3-methyl-1-(4-nitrophenyl)-2-benzopyrilium perchlorate (22). To an ice cold solution of **21** (6.8 g, 22.4 mmol) in acetone (70 mL) Jones reagent (29 mL, 78 mmol) was added dropwise during 1 h and the mixture was then stirred at rt for 4 h. The separated chromium salt was filtered and the solution evaporated to dryness. The residue was suspended with water (25 mL) and filtered. The precipitate was then dissolved in hot acetic acid (76 mL) and 70% perchloric acid (1.48 mL) was

Table 11. Physical and spectroscopic data of the 3-substitued 8-chloro-6-(4-nitrophenyl)-11*H*-1,2,4-triazolo[4,5-c][2,3]benzodiazepine derivatives **19a–d**

Compound	mp (°C)	Yield (%)	Molecular formula (Mol. mass)	Spectroscopic data: ^{1}H NMR or MS
19 a	271–274	72	$C_{17}H_{12}ClN_5O_2$ (353.8)	2.58 (s,3H) 4.16 (s, 2H), 7.12 (d, J = 1.9 Hz, 1H), 7.45 (d, J = 8.3 Hz, 1H), 7.55 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H), 7.90 (d, J = 8.7 Hz) and 8.35 (d, J = 8.7 Hz, nitrophenyl).
19 b	287–289	86	$C_{21}H_{13}ClN_6O_2$ (416.8)	(in DMSO-d_6) 4.46 (br. s, 2H), 7.24 (br. s, 1H), 7.78 (br. s, 2H), 7.98 (d, J = 8.8 Hz, 2H) and 8.42 (d, J = 8.8 Hz, 2H, nitrophenyl), 8.18 (d, J = 5.5 Hz, 2H) and 8.83 (d, J = 5.5 Hz, 2H, pyridyl).
19 c	287–290	68	$C_{22}H_{13}ClN_6O_4$(460.8)	MS(EI):m z: M:460,462.
19 d	266–268	88	$C_{18}H_{14}ClN_5O_3$(383.8)	MS(EI): m z: M: 383-385.

added. A product separated which was filtered after cooling and washed with acetic acid to give the title product. Yield: 42% (3.73 g). Mp 247–255 °C. $C_{16}H_{11}Cl_2NO_7$ (400.2). IR: 1096 cm^{-1} (ClO_4^-).

8-Chloro-4-methyl-1-(4-nitrophenyl)-5*H*-2,3-benzodiazepine (23). Compound **22** (4.1 g, 10.2 mmol) was added to a solution of 98% hydrazine hydrate (1.5 mL, 70.7 mmol) in DMF (20 mL) at 10–15 °C. The reaction mixture was then stirred for 1.5 h at rt Water (25 mL) was added and the separated precipitate was collected by filtration and washed with water. The crude product was recrystallized from isopropanol. Yield: 87% (2.82 g). Mp 199–203 °C. $C_{16}H_{12}ClN_3O_2$ (313.7). ^{1}H NMR: (DMSO-d_6): δ 2.11 (s, 3H, CH_3), 2.93 and 3.65 (d, J = 12.2 Hz, 2H, 5-H), 7.33 (d, J = 2.0 Hz, 1H, 9-H), 7.61 (d, J = 8.2 Hz, 1H, 6-H), 7.73 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 7-H), 7.82 (d, J = 8.7 Hz, 2H), 8.32 (d, J = 8.7 Hz, 2H).

8-Chloro-4-formyl-1-(4-nitrophenyl)-5*H*-2,3-benzodiazepine (24). To a solution of **23** (9.17 g, 29.0 mmol) in dioxane (120 mL) powdered selenium dioxide (2.27 g, 20.5 mmol) was given and the mixture was stirred at 90 °C for 40 min. Charcoal was added to the mixture and it was filtered. The clear solution was poured into water (1.5 L) and the separated crystalls were filtered and washed with water. Column chromatography with benzene as eluent gave the aldehyde. Yield: 29% (2.8 g). Mp 208–210 °C. $C_{16}H_{10}ClN_3O_3$ (327.7). ^{1}H NMR: (DMSO-d_6): 2.98 (d, J = 13.1 Hz) and 4.13 (d, J = 13.1 Hz, 2H, 5-H), 7.42 (d, J = 2.2 Hz, 1H, 9-H), 7.50 (d, J = 8.3 Hz, 1H, 6-H), 7.73 (dd, J_1 = 8.3 Hz, J_2 = 2.2 Hz, 1H, 7-H), 7.92 (d, J = 8.9 Hz, 2H) and 8.36 (d, J = 8.9 Hz, 2H, nitrophenyl).

6-(4-Aminophenyl)-8-chloro-3-methyl-11*H*-imidazolo[3,4-c][2,3]benzodiazepine (5). The solution of **24** (2.15 g, 6.60 mmol) in a 1:1 mixture of THF:water (88 mL) was chilled with icewater and sodium borohydride (0.12 g 3.30 mmol) was given in portions. After stirring at rt for 40 min the solution was diluted with water (90 mL) to give a precipitate which was filtered and after drying chromatographed with the eluent of benzene:ethyl acetate (1:1) to give pure 4-(hydroxymethyl)-8-chloro-1-(4-nitrophenyl)-5*H*-2,3-benzodiazepine (**25**; 1.62 g). Mp > 163 °C (decomposition). (MS(FAB): M: 329/331.)

The intermediate **25** (1.62 g, 4.9 mmol), triphenylphosphine (2.54 g, 9.7 mmol) and phthalimide (1.42 g, 9.7 mmol) were dissolved in dry THF (72 mL) and a solution of diethyl azodicarboxylate (1.52 mL, 9.7 mmol) in THF (11 mL) was added dropwise. After stirring for 3 h at rt the solvent was removed and the residue recrystallized from ethanol to give **26** (1.34 g). Mp 254–256 °C (dec.). (MS(FAB): M: 458,460.)

The solution of the intermediate **26** (1.34 g, 2.9 mmol) and 98% hydrazine hydrate (1.09 mL, 21.7 mmol) in methanol (134 mL) was heated at boiling for 4 h. After evaporation of the solvent the residue was treated with methanol (50 mL) and the resulting precipitate was filtered. The filtrate was then evaporated and the residue suspended with water to give **27** (0.97 g, mp 105–107 °C (dec.)). This crude intermediate was taken up with acetic anhydride (8 mL) and stirred for 2 h. Dilution with water (40 mL) gave a solid substance which was collected by filtration. Column chromatography of this product with the eluent ethyl acetate:benzene (4:1) gave the acetylamino derivative **28** (0.60 g). Mp 216–218 °C. (MS(FAB): M: 370,372.)

A solution of **28** (0.59 g, 1.6 mmol) and $POCl_3$ (0.73 mL, 7.95 mmol) in 1,2-dichloroethane (30 mL) was heated at reflux for 3 h. The solution was then chilled with ice and treated with sodium hydrogencarbonate solution. After separation the organic phase was washed with water, dried and evaporated. The residual oil was chromatographed with an eluent of ethyl acetate:benzene (4:1) to give 8-chloro-3-methyl-6-(4-nitrophenyl)-11*H*-imidazolo [3,4-c][2,3]benzodiazepine (**29**), which was then reduced according to the general procedure as described for compounds **3a–n**. The crude product was boiled with ethanol to remove impurities. By the above sequence 0.12 g of pure **5** could be prepared. Mp 256–258 °C (dec.). $C_{18}H_{15}ClN_4$ (322.8). MS(EI): *m/z* (%): M: 322/324 (100/33), 321/323 (26/9), 108.5 (22), 65 (11), 219 (10), 106 (10), 218 (8), 217 (8).

6-(4-Aminophenyl)-8-chloro-2-ethoxycarbonyl-11*H*-pyrrolo[1,2-c][2,3]benzodiazepine (6). The solution of **23** (0.50 g, 1.60 mmol) and ethyl bromopyruvate (0.27 mL, 2.20 mmol) in ethanol (20 mL) was heated at reflux for 12 h. After evaporation of the solvent the residue was chromatographed with benzene as eluent to give **30** (0.29 g) which was reduced without further purification according to the general procedure as described for **3a–n**. It was recrystallized from ethanol (0.11 g). Mp 247–249 °C. $C_{21}H_{18}ClN_3O_2$ (379.8). MS (EI): *m/z* (%): M: 379/381 (100/35), 378/380 (41/14), 350/352 (35/14), 306/308 (35/12), 65 (23).

General procedure for the reduction of the nitro compounds 15a–n, 19a–d, 29 and 30. (Preparation of 3a–n, 4a–d, 5 and 6). To a stirred solution of the corresponding nitro compound (2 mmol) in a 1:1 mixture of methanol and dichloromethane hydrazine hydrate (4–5 equiv) and RaNi (0.1–2 g) were added. After stirring at 20–40 °C for 1–5 h the catalyst was filtered and the solvents evaporated. The residues were recrystallized from ethanol. Mp-s and yields of the aminophenyl compounds **3a–n** and **4a–d** are shown in Table 1 and 2 respectively. The composition and spectroscopic data of **3a–3r** and **4a–d** are collected in Table 12.

6-(4-Acetylaminophenyl)-8-chloro-2-methyl-11*H*-imidazolo[1,2-c][2,3]benzodiazepine (3o). Prepared from **3b** (0.46 g, 1.42 mmol) in pyridine (8 mL) by reacting with acetylchloride (0.20 mL) at 5–10 °C for 1.5 h. Dilution with ice water gave a solid precipitate which was recrystallized from ethanol. For yield and mp see Table 1, spectroscopic data are shown in Table 12.

8-Chloro-3-methyl-6-phenyl-11*H*-imidazolo[1,2-c][2,3]benzodiazepine (3r). To a stirred solution of **3c** (1.10 g, 3.20 mmol) in DMF (12 mL) at 65 °C isoamyl nitrite (0.80 mL) was added droppwise over 8 min. Heating was continued for 30 min and the cooled mixture was then treated with 5N HCl. The product was extracted with ether and purified by column chromatography. Eluent: chloroform:methanol (98:2). For yield and mp see Table 1, spectroscopic data are shown in Table 12.

Biology, in vitro methods

Retinal spreading depression (S.D.) test. Experiments were performed according to Sheardown.[17] The posterior chamber of each eye of 1–5-day-old chickens was dissected and placed in a Petri dish containing physiological saline of the following composition: NaCl (100 mM), KCl (6 mM), $CaCl_2$ (1 mM), $MgSO_4$ (1 mM), $NaHCO_3$ (30 mM), NaH_2PO_4 (1 mM). The solution was saturated with 95% O_2 and 5% CO_2 and maintained at 26 °C. The eyes were initially incubated in normal saline for 30 min and then transferred to a solution containing AMPA (5 μM) or kainate (5 μM). These solutions triggered S.D., which could be easily observed by eye. The latency of S.D. was determined. The presence of a white area (0.5 mm in diameter) was taken as the onset of S.D. The eye cups were then returned to normal saline. After a further 15 min recovery period the eyes were incubated in a solution containing the test compounds and incubated for 15 min. Thereafter the eyes were transferred to a glutamate agonist containing solution which also contained the test compound and the latency of S.D. was determined again. 60 min following this measurement, the whole procedure was repeated, with another test substance/concentration. Each drug concentration was tested in 6 retina preparations. An increase in the latency of 30 s or more was considered to be 100% inhibition of S.D. (cut-off time was 1 min).[17] The drug effects therefore are expressed as the percentage maximum inhibition obtained for a given concentration. Dose-response curves were constructed from 3–6 concentration points and IC_{50}±S.E.M. values were calculated by sigmoidal curve fitting using the MICROCAL ORIGIN 4.1 computer program.

Electrophysiological studies. Electrophysiological experiments were carried out on acutely isolated cerebellar Purkinje cells according to Bleakman et al.[19] Briefly: cells were isolated from the cerebellum of 6–9-day-old rats using enzymatic treatment and mechanical trituration, then plated to poly-L-lysine coated glass coverslips. Cells were kept alive in a tissue culture medium, in a CO_2 thermostat, and used for electrophysiological experiments on the day of isolation. Purkinje cells (identified by their bigger size) were patched with glass electrodes (4–6 MΩ). The membrane potential was fixed at −70 mV. Experiments were performed at room temperature. The internal recording solution contained NaCl (140 mM), $MgCl_2$ (1 mM), HEPES (10 mM), glucose (10 mM), EGTA (0.1 mM), pH = 7.2. The cells were continuously perfused with a solution composed of NaCl (138 mM), KCl (5 mM), $CaCl_2$ (5 mM), $MgCl_2$ (1 mM), HEPES (10 mM), glucose (10 mM), pH = 7.35. Excitatory amino acids (kainate 100 μM or S-AMPA 5 μM) were applied alone, or in combination with various concentrations of **1**, **2** or **3b**. The percentage decrease of the plateau current was evaluated. IC_{50}±S.E.M. values were calculated from 4–6 concentrations (5–6 cells for each concentration), using the MICROCAL ORIGIN 4.1 computer program for sigmoidal curve fitting.

In vivo experiments

Animals were purchased from Charles River Hungary Ltd. (Budapest, Hungary) and were housed for a minimum of six days prior to experiments with free access to standard laboratory diet and tap water and maintained on a 12–12 h light-dark cycle (light from 6.00 am to 6.00 p.m.). All compounds under study were suspended in saline (ip administration) or distilled water (oral administration) containing 1–2% Tween-80. Control animals received vehicle under the same conditions. ED_{50} and LD_{50} values were calculated by the Litchfield–Wilcoxon method.[34] The volumes of administration were 0.1 mL/10 g and 0.5 mL/100 g body weight for mice and rats, respectively, unless otherwise stated.

Behavioural changes in mice. 5 male CD1 mice (21–26 g) were starved for 16 h and treated with test compounds in doses of 100 and 200 mg/kg ip and po, respectively. The gross behavioural changes were evaluated continuously for 5 h according to Irwin.[35]

Seizure assays. 10 male CD1 mice (21–26 g) per group, after 16 h starvation, were used. Animals were treated orally with test compounds 60 min before maximal electroshock (MES)[36] or chemical convulsants (metrazole 130 mg/kg ip, strychnine 3 mg/kg ip, bemegride 50 mg/kg ip, bicuculline 1 mg/kg iv, nicotine 3.5 mg/kg iv, 4-aminopyridine 12.5 mg/kg ip and 3-mercapto-propionic acid 110 mg/kg iv).[37] Prevention of tonic convulsion (MES) and/or death (convulsive agents) served as measure of anticonvulsive potency.

Muscle relaxation in mice. (Inclined screen and rotarod tests). For determination of muscle relaxant activity the inclined screen test[38] and rotarod test[39] were used. 10 male CD1 mice (21–25 g) per group were examined.

Table 12. Composition and spectroscopic data of compounds 3a–r and 4a–d

Compound	Molecular formula (Mol. mass)	Spectroscopic data: ^{1}H NMR (in DMSO-d_6) or MS
3a	$C_{17}H_{13}ClN_4$ (308.8)	4.00 (br. s, 2H, 11-H), 5.85 (br. s, NH_2), 6.84 and 7.38 (d, J = 1.5 Hz, 2-H and 3-H), 6.63 (d, J = 8.7 Hz, 2H) and 7.36 (d, J = 8.7 Hz, 2H, aminophenyl), 7.21 (d, J = 2.0 Hz, 1H, 7-H), 7.58 (d, J = 8.3 Hz, 1H, 10-H), 7.63 (dd, J_1 = 8.3 Hz, J_2 = 2.0 Hz, 1H, 9-H).
3b	$C_{18}H_{15}ClN_4$(322.8)	2.04 (d, J = 1.1 Hz, 1H, 2-CH_3), 3.91 (br. s, 2H, 11-H), 5.80 (br. s, 2H, NH_2), 6.63 (d, J = 8.7 Hz, 2H) and 7.37 (d, J = 8.7 Hz, 2H, aminophenyl), 7.07 (q, J = 1.1 Hz, 1H, 3-H), 7.19 (d, J = 2.1 Hz, 1H, 7-H), 7.56 (d, J = 8.3 Hz, 2H, 10-H), 7.64 (dd, J_1 = 8.3 Hz, J_2 = 2.1 Hz, 1H, 9-H).
3c	$C_{18}H_{15}ClN_4$(322.8)	(in $CDCl_3$) 2.32 (d, J = 1.0 Hz, 3H, 3-CH_3), 3.92 (br. s, 2H, 11-H), 4.08 (br. s, 2H, NH_2), 6.63 (q, J = 1.0 Hz, 1H, 2-H), 6.75 (d, J = 8.5 Hz, 2H) and 7.55 (d, J = 8.5 Hz, 2H, aminophenyl), 7.29 (d, J = 1.9 Hz, 1H, 7-H), 7.36 (d, J = 8.1 Hz, 1H, 10-H), 7.44 (dd, J_1 = 8.1 Hz, J_2 = 1.9 Hz, 1H, 9-H).
3d	$C_{19}H_{17}ClN_4$(336.8)	2.00 and 2.18 (s, 3H, CH_3), 3.90 (br. s, 2H, 11-H), 5.82 (br. s, 2H, NH_2), 6.68 (d, J = 8.8 Hz, 2H) and 7.42 (d, J = 8.8 Hz, 2H, aminophenyl), 7.20 (d, J = 1.9 Hz, 1H, 7-H), 7.54 (d, J = 8.3 Hz, 1H, 10-H), 7.64 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H, 9-H).
3e	$C_{19}H_{17}ClN_4$(336.8)	(in $CDCl_3$) 1.26 (t, J = 7.3 Hz, 3H), 2.6 (q, J = 7.3 Hz, 2H), 3.94 (br. s, 2H, 11-H), 6.72 (d, J = 8.6 Hz, 2H) and 7.52 (d, J = 8.6 Hz, 2H, aminophenyl), 6.97 (s, 1H, 3-H), 7.27 (d, J = 2.0 Hz, 1H, 7-H), 7.34 (d, J = 8.2 Hz, 1H, 10-H), 7.45 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 9-H).
3f	$C_{23}H_{18}ClN_5$(399.9)	3.6-4.3 (2H, 11-H), 5.25 (br. s, 2H) and 5.86 (br. s, 2H, NH_2), 6.64 (d, J = 8.8 Hz, 2H), 6.66 (d, J = 8.8 Hz, 2H) and 7.27 (d, J = 8.8 Hz, 2H), 7.35 (d, J = 8.8 Hz, 2H, aminophenyls), 7.30 (d, J = 1.9 Hz, 1H, 7-H), 7.60 (d, J = 8.3 Hz, 1H, 10-H), 7.65 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H, 9-H).
3g	$C_{22}H_{16}ClN_5$(385.9)	3.8-4.3 (2H, 11-H), 7.30 (d, J = 2.0 Hz, 1H, 7-H), 7.46 (s, 1H, 2-H), 7.62 (d, J = 8.2 Hz, 1H, 10-H), 7.67 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 9-H), 6.68 (d, J = 8.6 Hz, 2H) and 7.40 (d, J = 8.6 Hz, 2H, aminophenyl), 7.68 (d, J = 5.6 Hz, 2H) and 8.62 (d, J = 5.6 Hz, 2H, pyridyl).
3h	$C_{23}H_{17}ClN_4$(384.9)	MS (EI): m/z (%): M: 384/386 (100/33).
3i	$C_{18}H_{14}Cl_2N_4$(357.2)	(in $CDCl_3$) 2.22 (d, J = 1.0 Hz, 3H, 2-CH_3), 3.96 (br. s, 2H, 11-H), 4.08 (br. s, 2H, NH_2), 6.98 (q, J = 1.0 Hz, 1H, 3-H), 6.65 (d, J = 8.0 Hz, 2H) and 7.53 (d, J = 8.6 Hz, 2H, aminophenyl), 7.30 (s, 1H), 7.39 (s, 1H).
3j	$C_{18}H_{14}Cl_2N_4$(357.2)	2.21 (d, J = 1.0 Hz, 3H, 3-CH_3), 4.00 (br. s, 2H, 11-H), 6.60 (q, J = 1.0 Hz, 1H, 2-H), 6.68 (d, J = 8.6 Hz, 2H) and 7.45 (d, J = 8.6 Hz, 2H, aminophenyl), 7.40 (s, 1H), 7.93 (s, 1H).
3k	$C_{18}H_{15}BrN_4$(367.3)	2.02 (d, J = 1.2 Hz, 3H, 2-CH_3), 3.90 (br. s, 2H, 11-H), 5.90 (br. s, 2H, NH_2), 7.07 (q, J = 1.2 Hz, 1H, 3-H), 6.62 (d, J = 8.5 Hz, 2H) and 7.35 (d, J = 8.5 Hz, 2H, aminophenyl), 7.32 (d, J = 2.2 Hz, 1H, 7-H), 7.47 (d, J = 8.3 Hz, 1H, 10-H), 7.75 (dd, J_1 = 8.2 Hz, J_2 = 2.2 Hz, 1H, 9-H).
3l	$C_{18}H_{15}BrN_4$(367.3)	2.22 (d, J = 1.0 Hz, 3H, 3-CH_3), 3.92 (br. s, 2H, 11-H), 5.82 (br. s, 2H, NH_2), 6.55 (q, J = 1.0 Hz, 1H, 2-H), 6.65 (d, J = 8.6 Hz, 2H) and 7.42 (d, J = 8.6 Hz, 2H, aminophenyl), 7.32 (d, J = 1.8 Hz, 1H, 7-H), 7.50 (d, J = 8.0 Hz, 1H, 10-H), 7.72 (dd, J_1 = 8.0 Hz, J_2 = 1.8 Hz, 1H, 9-H).
3m	$C_{19}H_{17}BrN_4$(381.3)	1.98 (s, 3H) and 2.16 (s, 3H, CH_3), 3.85 (br. s, 2H, 11-H), 5.80 (s, 2H, NH_2), 6.65 (d, J = 8.6 Hz, 2H) and 7.34 (d, J = 8.6 Hz, 2H, aminophenyl), 7.31 (d, J = 1.9 Hz, 1H, 7-H), 7.45 (d, J = 8.2 Hz, 1H, 10-H), 7.75 (dd, J_1 = 8.2 Hz, J_2 = 1.9 Hz, 1H, 9-H).
3n	$C_{22}H_{16}BrN_5$(430.3)	3.7–4.3 (br. 2H, 11-H), 5.95 (br. s, 2H, NH_2), 6.67 (d, J = 8.6 Hz, 2H) and 7.40 (d, J = 8.6 Hz, 2H, aminophenyl), 7.47 (s, 3H, 2-H), 7.45 (d, J = 2.0 Hz, 1H, 7-H), 7.57 (d, J = 8.2 Hz, 1H, 10-H), 7.78 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, 9-H), 7.71 (d, J = 5.6 Hz, 2H) and 8.63 (d, J = 5.6 Hz, 2H, pyridyl).
3o	$C_{20}H_{17}ClN_4O$(364.8)	(in $CDCl_3$) 2.18 (d, J = 1.0 Hz, 3H, 2-CH_3), 2.23 (s, 3H, acetyl), 3.94 (br. s, 2H, 11-H), 6.98 (q, J = 1.0 Hz, 1H, 3-H), 7.18 (d, J = 2.2 Hz, 1H, 7-H), 7.31 (d, J = 8.2 Hz, 1H, 10-H), 7.46 (dd, J_1 = 8.2 Hz, J_2 = 1.9 Hz, 1H, 9-H), 7.63 and 7.68 (d, J = 8.8 Hz, acetylaminophenyl), 8.13 (br. s, 1H, NH-Ac).
3p [a]	$C_{18}H_{14}BrCl_2N_3$(423.1)	2.32 (d, J = 1.0 Hz, 3H, 2-CH_3), 4.48 (br. s, 2H, 11-H), 7.12 (q, J = 1.0 Hz, 1H, 3-H), 7.55–7.80 (m, 5H), 7.90–8.00 (m, 2H).
3q [a]	$C_{18}H_{14}BrCl_2N_3$(423.1)	2.35 (d, J = 1.0 Hz, 3H, 3-CH_3), 4.45 (br. s, 2H, 11-H), 7.08 (d, J = 2.1 Hz, 1H, 7-H), 7.43 (q, J = 1.0 Hz, 1H, 2-H), 7.58 (d, J = 8.3 Hz, 1H, 10-H), 7.60–7.73 (m, 4H), 7.95 (dd, J_1 = 8.3 Hz, J_2 = 2.1 Hz, 1H, 9-H).
3r	$C_{18}H_{14}ClN_3$(307.8)	2.30 (br. s, 3H, CH_3), 4.05 (br. s, 2H, 11-H), 6.63 (br. s, 1H, 2-H), 7.12 (d, J = 1.9 Hz, 1H, 7-H), 7.50–7.85 (m, 7H).
4a	$C_{17}H_{14}ClN_5$(323.8)	2.40 (br. s, 3H, 3-CH_3), 4.13 (br, 2H, 11-H), 5.94 (br, 2H, NH_2), 6.64 (d, J = 8.6 Hz, 2H) and 7.43 (d, J = 8.6 Hz, 2H, aminophenyl), 7.22 (br. s, 1H, 7-H), 7.65 (br. s, 2H, 9-H and 10-H).
4b	$C_{21}H_{15}ClN_6$(386.8)	3.95–4.6 (br, 2H, 11-H), 6.67 (d, J = 8.6 Hz, 2H) and 7.42 (d, J = 8.6 Hz, 2H, aminophenyl), 7.28 (br. s, 1H, 7-H), 7.68 (br. s, 2H, 9-H and 10-H), 8.02 (br. s, 2H, pyridyl), 8.82 (br. s, 2H, pyridyl).
4c	$C_{22}H_{17}ClN_6$(400.9)	(in $CDCl_3$) 4.12 (br, 2H, 11-H), 6.72 (d, J = 8.6 Hz, 2H), 6.79 (d, J = 8.6 Hz, 2H) and 7.54 (d, J = 8.6 Hz, 2H), 7.84 (d, J = 8.6 Hz, 2H, aminophenyls), 7.32 (d, J = 2.1 Hz, 1H, 7-H), 7.40 (d, J = 8.2 Hz, 1H, 10-H), 7.50 (dd, J_1 = 8.2 Hz, J_2 = 2.1 Hz, 1H, 9-H)
4d	$C_{18}H_{16}ClN_5O$(353.8)	3.35 (s, 3H, OCH_3), 4.20 (br. s, 2H, 11-H), 4.62 (s, 2H, O-CH_2), 5.98 (br. s, 2H, NH_2), 6.63 (d, J = 8.7 Hz, 2H) and 7.42 (d, J = 8.7 Hz, 2H, aminophenyl), 7.22 (br. s, 1H, 7-H), 7.62 (br. s, 2H, 9-H and 10-H).

[a]Hydrochloride salts.

They were treated ip 30 min before testing. In the inclined screen test the untrained mice were placed on a screen inclining 60°. The number of falling mice was noted within 60 s test period and scored as positive. In the rotarod test mice were trained to do coordinated motor movements for 120 s on the rod (3.5 cm diameter) rotating at 15 rpm. The impairment of coordinated motor movements was defined as inability of selected mice to remain on the rotating rod for 120 s test period.

Transient occlusion of the middle cerebral artery (MCA) in rats. Male CD RB rats, weighing 370–410 g, were

used. The anaesthesia was induced by 5% and maintained by 2% halothane in a mixture of 30% N_2O and 70% O_2. The rectal body temperature was controlled and kept at 37 °C throughout the experimental period using a thermostatically controlled blanket.

The occlusion of the MCA was achieved as follows:[22,23] the left common carotid artery (CCA), the extracranial external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a midline incision. The branches of the ECA were then isolated and coagulated. The pterygopalatine artery, this posteriorly directed extracranial branch of the ICA, was occluded by a microvascular clip. Next a silk suture was tied loosely around the mobilised ECA stump which was cut at its first branching (leaving a stump about 2–3 mm) and used to introduce the embolus. The CCA was occluded by microvascular clip and a 5 cm length of 4–0 monofilament nylon suture, its tip rounded by heating near a flame, was introduced into the ECA lumen (22 mm distal to the carotid bifurcation). This was secured in place by tightening the silk ligature. At this point the intraluminal suture has blocked the origin of the MCA occluding of blood flow from the ICA, anterior and posterior cerebral arteries.

60 min after embolization a second short and light anaesthesia was induced by face mask. The nylon suture and the microvascular clip on the CCA were carefully removed, so the blood-flow from the CCA into the ICA was restored. 24 h after reperfusion the rats were decapitated. Their brains were rapidly removed 5 mm from the frontal pole 3 coronal sections (2 mm thick) were cut out from the cerebrum, immersed in a 2% solution of TTC for vital staining and placed in a 37 °C water-bath for 10 minutes and fixed in 10% formalin solution. TTC stained the intact areas of brain to deep red colour, but did not stain the infarcted tissue.

From each animal three brain slices were obtained with 2-fold magnification by using a copy machine. The areas of ischaemic damages were measured from these black-white pictures by a computerised scanning method (ARTEC SCAN A400).

Antagonism of oxotremorine-induced tremor in mice. 10 male CD1 mice (21–25 g) per group were starved for 16 h, then treated orally with test compounds or vehicle. 60 min later they received oxotremorine 10 mg kg ip[40] The intensity of tremor caused by oxotremorine was scored (0–0.5–1–2–3) and noted in every 5 min for 30 min. The scores were summed individually, means and SEM were calculated.

MPTP induced dopaminergic neurotoxicity. Male C57 black mice, weighing 23–30 g were used. Three days after a single ip injection of MPTP (30 mg kg) and four consecutive injections of the test compound (see Table 8 for the treatment schedule for each compound) mice were killed by decapitation. Both striata were dissected and kept at −80 °C until assayed. Striata were weighed and then sonicated in 200 µl of distilled water. Aliquots (80 µl) of the homogenates were resonicated in 80 µl of HPLC mobile phase containing α-methyl-dopamine as internal standard. The samples were centrifuged (10,000×g for 15 min at 4 °C) and a 5 µl aliquots were injected into the HPLC system. Calculated values are expressed as µg g wet tissue.

The concentrations of dopamine, DOPAC, HVA, 5-HT and 5-HIAA in the samples were determined by HPLC/electrochemistry according to Patthy and Gyenge.[41] The HPLC system used in this study consisted of a Knauer 64 isocratic pump with a pulse dampener, a BAS Unijet microbore injector equipped with a 5 µl sample loop, a BAS microbore ODS 3 µm (100×1 mm) analytical column. For electrochemical detection, glassy carbon electrodes (BAS LC-4C) set at +0.75 V against an Ag AgCl reference electrode were used. The mobile phase composition was aqueous buffer:acetonitrile (95:5) (v/v). The aqueous buffer consisted of 3 mM HFBA (heptafluorobutyric acid) as pairing ion, 100 mM sodium hydroxide, 0.1 g l Na_2EDTA, and the pH was adjusted to 4.3 with 42.5% (w/v) phosphoric acid. All other chemicals used were of analytical reagent grade. One-way analysis of variance (ANOVA) followed by the Duncan's multiple range test was used for statistical analysis.

Acute toxicity in mice. The LD_{50} values were calculated from lethality within 14 days after ip and po administration of the drug.[42]

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Autoimmune encephalomyelitis ameliorated by AMPA antagonists

TERENCE SMITH, ANTHONY GROOM, BIN ZHU & LECHOSLAW TURSKI

Eisai London Research Laboratories, Bernard Katz Building, University College London, Gower Street, London WC1E 6BT, UK
Correspondence should be addressed to L.T. or T.S.; email: lechoslaw.turski@solvay.com or terence_smith@eisai.net

Multiple sclerosis is an immune-mediated disorder of the central nervous system leading to progressive decline of motor and sensory functions and permanent disability[1,2]. The therapy of multiple sclerosis is only partially effective, despite anti-inflammatory, immunosuppresive and immunomodulatory measures[3]. White matter inflammation and loss of myelin, the pathological hallmarks of multiple sclerosis, are thought to determine disease severity[4,5]. Experimental autoimmune encephalomyelitis reproduces the features of multiple sclerosis in rodents and in nonhuman primates[6,7]. The dominant early clinical symptom of acute autoimmune encephalomyelitis is progressive ascending muscle weakness[6]. However, demyelination may not be profound and its extent may not correlate with severity of neurological decline[8], indicating that targets unrelated to myelin or oligodendrocytes may contribute to the pathogenesis of acute autoimmune encephalomyelitis. Here we report that within the spinal cord in the course of autoimmune encephalomyelitis not only myelin but also neurons are subject to lymphocyte attack and may degenerate. Blockade of glutamate AMPA receptors ameliorated the neurological sequelae of autoimmune encephalomyelitis, indicating the potential for AMPA antagonists in the therapy of multiple sclerosis.

To induce experimental autoimmune encephalomyelitis (EAE), we immunized Lewis rats subcutaneously in both hind feet with inoculum containing guinea pig myelin basic protein emulsified in Freund's complete adjuvant containing *Mycobacterium tuberculosis*. Neurological assessments demonstrated progressive development of tail and hindlimb paralysis, leading to paraplegia and loss of the righting reflex. The neurological decline started 11 days (11.13 ± 0.21; $n = 40$) after immunization, peaked after 13 days (13.08 ± 0.25), and waned after 16 days (15.75 ± 0.22) (Fig. 1). Rats showed a progressive loss of as much as 21% in body weight (maximal weight before onset, 207.74 ± 1.99 g compared with before, minimal weight after onset, 163.99 ± 2.22 g) starting 10 days (9.78 ± 0.21; $n = 40$) after immunization.

Morphological analysis by light and electron microscopy showed the presence of inflammation throughout lumbar spinal cord, with characteristic perivascular cuffs, lymphocytes and macrophages in white and gray matter. Demyelination was limited to root entry and exit zones. There was some edema in the white matter of dorsal columns at the peak of neurological symptoms.

Ultrastructurally, lymphocytes initially approached motoneurons, attached to their membranes (Fig. 2*a*) and were subsequently internalized (Fig. 2*b*). Integrated lymphocytes within neurons appeared morphologically normal and were surrounded by a membrane forming a vacuolar structure (Fig. 2*b*). Nuclear chromatin in lymphocytes trapped in motoneurons formed clumps attached to the nuclear membrane. After the disintegration of the nuclear membranes, lymphocytic nucleoplasm and cytoplasm intermixed and apoptotic bodies were formed. At late stages of lymphocyte breakdown within intraneuronal vacuoles, cell masses and the apoptotic bodies were transformed into amorphous debris (Fig. 2*c*). As the process of sequestration of lymphocytes into motoneurons continued, the neuronal cytoplasm became overwhelmed, whereas the nucleus remained intact (Fig. 2*c*). At the end of this process, motoneurons decomposed and formed large circular vacuoles filled with apoptotic lymphocytes and amorphous cellular debris (Fig. 2*d*). Analysis of the outer membranes of cells filled with lymphocytes showed that synaptic densities and presynaptic endings were preserved (Fig. 2*b* and *c*, insets), confirming their neuronal origin. The appearance in the spinal cord of neuronal vacuoles containing lymphocytes undergoing apoptosis correlated with clinical stages of the disease, with few vacuoles being detected at disease onset and the numbers increasing concomitant with highest disability scores and decreasing on recovery.

Morphometric analysis showed that the density of neurons in the ventral horns of the lumbar spinal cord decreased during the course of EAE by 30% (Table 1). There were no changes in the density of neurons in the intermediate and dorsal portions of the lumbar spinal cord (Table 1).

To determine whether non-neuronal cells were involved in the interaction with lymphocytes, we used immunohistochemistry for glial fibrillary acidic protein to identify astrocytes. In spinal cord sections from rats undergoing EAE at the peak of motor decline, cells positive for glial fibrillary acidic protein were distinct from those that contained lymphocytes (Fig. 3*c*). To determine the cell type entering neurons during acute EAE, we treated spinal cord sections with monoclonal antibodies specific

Table 1 Density of neurons in the lumbar spinal cord of rats subjected to EAE, and the effect of NBQX on cell loss in the ventral horns

	Density of neurons (N_v)						
Treatment	Ventral horns	%	intermediate zone	%	Dorsal horns	%	n
Sham + Vehicle	11,746 ± 408	100	38,952 ± 820	100	77,747 ± 1,353	100	7
EAE + Vehicle	8,264 ± 386*	70	36,934 ± 2,056	95	72,098 ± 2,701	93	8
EAE + NBQX	10,250 ± 715**	87	39,040 ± 1,685	100	76,983 ± 2,661	99	8

Neuronal loss was estimated 13–16 days after immunization with myelin basic protein. Data represent mean ± s.e.m. cells/mm³. N_v, numerical density. *$P < 0.001$, compared with sham-immunized, vehicle-treated rats; **, $P < 0.05$, compared with immunized, vehicle-treated rats; Student's *t*-test.

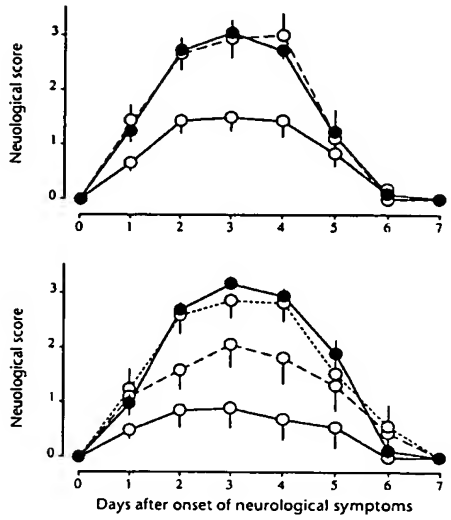

Fig. 1 Dose–response relationship of AMPA/kainate antagonists on motor disability induced by autoimmune encephalomyelitis in rats. NBQX, MPQX or vehicle were administered intraperitoneally twice daily for 7 d starting on day 10 after immunization. *a*, O, NBQX at a dose of 3 mg/kg (n = 10; dashed line) or 30 mg/kg (n = 30; solid line); ●, vehicle (n = 40). NBQX reduced disability in rats subjected to EAE ($F(3,76) = 14.86$, $P < 0.001$, analysis of variance with multiple comparisons). NBQX at a dose of 30 mg/kg reduced disability compared with vehicle ($F(1,62) = 36.24$, $P < 0.001$) and either 3 mg/kg NBQX ($F(1,31) = 32.54$, $P < 0.01$) or 10 mg/kg NBQX ($F(1,29) = 32.94$, $P < 0.01$) (n = 10), whereas 3 and 10 mg/kg NBQX did not differ from each other or vehicle. Onset of neurological symptoms was not affected by 3–30 mg/kg NBQX, whereas 30 mg/kg NBQX shortened duration of the disease ($P < 0.001$, Fisher's protected least-significant-difference test). *b*, O, MPQX at a dose of 2.5 mg/kg (n = 10; dotted line), 5 mg/kg (n = 10; dashed line), 10 mg/kg (n = 12; solid line); ●, vehicle (n = 26). MPQX reduced disability in rats subjected to EAE in a dose-dependent manner ($F(3,42)=13.40$, $P < 0.001$). MPQX at a dose of 10 mg/kg reduced disability compared with vehicle ($F(1,27) = 55.83$, $P < 0.001$) and 2.5 mg/kg MPQX ($F(1,15) = 54.97$, $P < 0.001$), whereas 2.5 and 5 mg/kg MPQX did not differ from each other or vehicle. Onset of neurological symptoms was not affected by 2.5–10 mg/kg MPQX, whereas 10 mg/kg MPQX shortened duration of the disease ($P < 0.001$, Fisher's protected least-significant-difference test). Data represent means ± s.e.m.

for T, B, and natural killer lymphocytes, or macrophages/microglia. The cells entering neurons during the acute phase of EAE stained positive for CD2 but not for CD45RA, CD161 or ED1, showing that T lymphocytes were involved and not B or natural killer lymphocytes, or macrophages/microglia (Fig. 3*a* and *b*).

Spinal motoneurons have high densities of glutamate receptors and are sensitive to toxicity mediated by glutamate through non-NMDA (N-methyl-D-aspartate) receptors[9]. Activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate receptors mediates fast neurotransmission in the brain and spinal cord[10]. Administration of AMPA or kainate into the rat lumbar cord rapidly precipitates hindlimb weakness[11].

To determine whether AMPA/kainate receptor antagonists affect the clinical and morphological outcome of acute EAE, we administered two competitive antagonists, NBQX (ref. 12) and MPQX (ref. 13), and two noncompetitive antagonists, GYKI52466 and GYKI53773 (ref. 14), intraperitoneally to immunized rats. Treatment with 30 mg/kg NBQX twice daily for 7 days starting on day 10 after immunization improved neurological outcome, reducing the cumulative score from 11.06 ± 0.48 in vehicle-treated rats (n = 40) to 5.85 ± 0.98 in NBQX-treated rats (n = 30)($P < 0.001$, Mann-Whitney U test) (Fig. 1*a*) and limiting neuronal death in the spinal cord (Table 1). NBQX at a dose of 30 mg/kg also decreased weight loss during the course of EAE by 19% (43.75 ± 1.19 g in vehicle-treated rats compared with 35.39 ± 1.84 g in NBQX-treated rats; $P < 0.001$, Student's t-test), but had no effect on weight loss at doses of 10 mg/kg (n = 10) and 3 mg/kg (n = 10). Treatment with 10 mg/kg MPQX twice daily for 7 days starting on day 10 after immunization reduced the cumulative disease score from 10.95 ± 0.56 in vehicle-treated rats (n = 26) to 3.15 ± 1.29 in MPQX-treated rats (n = 16)($P < 0.001$, Mann-Whitney U test). MPQX given twice daily at a dose of 5 mg/kg also reduced disease severity, decreasing the cumulative score to 8.35 ± 1.91 (n = 16), but it had no effect at a dose of 2.5 mg/kg (11.60 ± 1.43; n = 10) (Fig. 1*b*). MPQX at doses of 2.5–10 mg/kg had no effect on weight loss. GYKI52466 given to rats intraperitoneally at a dose of 40 mg/kg at 0, 6, 12, 18 and 24 hours on day 10 after immunization improved the clinical outcome, reducing the cumulative score from 13.81 ± 0.58 in vehicle-treated rats (n = 13) to 9.51 ± 0.55 in GYKI52466-treated rats (n = 12)($P < 0.001$, Mann-Whitney U test). Treatment with 30 mg/kg GYKI52466 or GYKI53773, twice daily for 7 days starting on day 10 after immunization, improved the neurological outcome, reducing the cumulative score from 9.53 ± 1.71 (n = 9) in vehicle-treated rats to 6.90 ± 1.37 in GYKI52466-treated rats (n = 10) or 2.13 ± 0.70 in GYKI53773-treated rats (n = 10)($P < 0.001$, Mann-Whitney U test). Treatment with 30 mg/kg GYKI52466 or GYKI53773 had no effect on weight loss.

To determine whether AMPA antagonists interact with the induction of immunization, we administered 30 mg/kg NBQX intraperitoneally to rats twice daily for 7 days starting 4 days after immunization. This treatment regimen did not affect the cumulative clinical score (11.40 ± 1.22, vehicle-treated (n = 10); 9.38 ± 0.82, NBQX-treated (n = 10); not significant, Mann-Whitney U test) or decrease weight loss compared with that of vehicle-treated rats. We also used adoptive transfer of EAE in rats[4] to determine whether AMPA antagonists interact with immunization. We transferred myelin basic protein-sensitized splenic lymphocytes from immunized rats to naive rats and began treatment with 30 mg/kg NBQX twice daily for 7 days on day 4 after transfer. Seven of eight rats given vehicle alone showed disability ranging from flaccid tail to hindlimb weakness, whereas one of eight rats treated with NBQX showed mild changes in tail tone and none showed motor disability ($P < 0.005$, compared with vehicle-treated rats, χ^2-test).

To determine whether NBQX mitigates the CNS inflammation triggered by immunization, we analyzed sections of rat brainstem for the presence of perivascular cuffs. Treatment with 30 mg/kg NBQX twice daily for 7 days starting on day 10 after immunization improved the neurological outcome, reducing the cumulative scores from 10.33 ± 0.56 in vehicle-treated rats (n = 17) to 4.04 ± 1.04 in NBQX-treated rats (n = 17) ($P < 0.001$, Mann-Whitney U test). However, this treatment did not affect inflammation (histopathological scores: 166.94 ± 23.26 in vehicle-treated rats and 118.59 ± 14.96 in NBQX-treated rats; not significant, Mann-Whitney U test).

To assess whether AMPA antagonists improved disability in EAE because of immunosuppression, we studied the effects of NBQX, MPQX and of the glucocorticoid dexamethasone on immunogen-induced lymphocyte proliferation. Neither NBQX nor MPQX, at concentrations up to 100 μM, influenced incorpora-

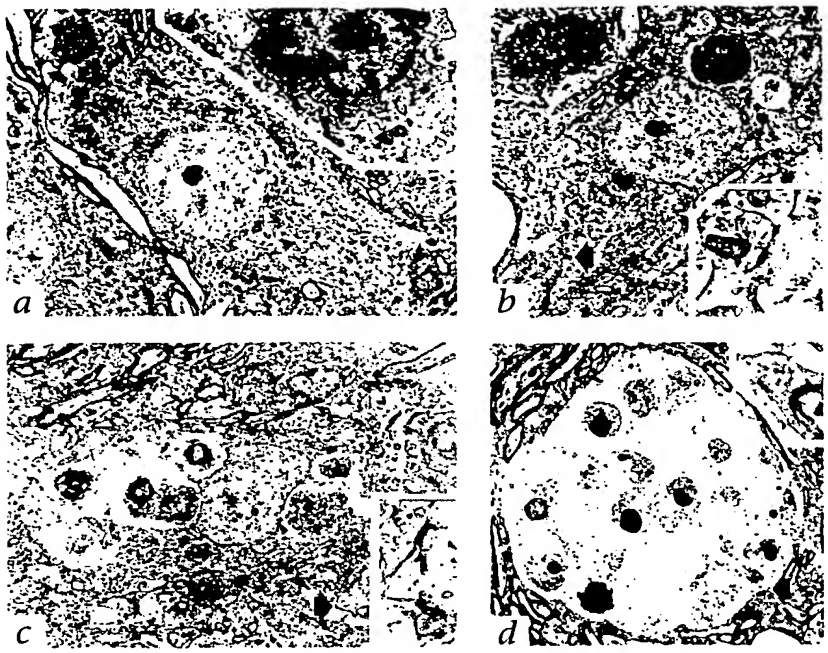

Fig. 2 Electron micrographs of the ventral horn of the lumbar spinal cord from Lewis rats immunized with myelin basic protein, at the peak of the disease course, showing different stages of degeneration of motoneurons associated with lymphocyte entry. *a*, A lymphocyte (magnified in inset) is attached to the membrane of the motoneuron. *b*, A lymphocyte that has invaded the motoneuron is entirely engulfed by its cytoplasmic membrane and is beginning to undergo apoptosis. The host motoneuron seems intact. *c*, Several lymphocytes have entered the motoneuron, are filling up its cytoplasm and are in different stages of apoptosis. *d*, A decomposed motoneuron forms a vacuole filled with lymphocytes and amorphous cellular debris. The presence of synaptic densities on the cytoplasmic membranes (arrows; magnified in insets, *b*, *c* and *d*) identifies the cells as neurons. Original magnifications: × 3,500 (*a*), × 15,210 (*a*, inset), × 5,232 (*b*), × 35,900 (*b*, inset), × 3,486 (*c*), × 34,620 (*c*, inset), × 3,815 (*d*) and × 50,880 (*d*, inset).

tion of thymidine into T lymphocytes, whereas dexamethasone was effective, with an ED_{50} (dose required ti suppress thymidine incorporation by 50%) of 0.43 μM (range, 0.32–0.59 μM.

To determine whether AMPA/kainate antagonists affect clinical outcome of chronic relapsing EAE (ref. 6), we administered NBQX intraperitoneally to immunized mice. Treatment with 30 mg/kg NBQX twice daily for 7 days starting on day 10 after immunization improved the neurological outcome, reducing disease severity between days 10 and 48 after immunization ($F(1,38)=9.21$; $P < 0.001$) (Fig. 4*a*). Treatment with 30 mg/kg NBQX once daily for 17 days beginning on day 26 after immunization also reduced disease severity between days 28 and 48 after immunization ($F(1,20)=2.76$; $P < 0.05$) (Fig. 4*b*).

Ultrastructural analysis of the spinal cord in rats has indicated involvement of neurons as well as non-neuronal cell populations (lymphocytes, macrophages and oligodendrocytes) in the pathogenesis of EAE. Lymphocytes initiate inflammation during EAE, and the degree of infiltration of spinal cord by T lymphocytes correlates with the time course of the disease[6]. T lymphocytes may approach and enter neurons during acute EAE. Temporary internalization of a morphologically normal cell by another cell without morphological or functional harm to both host and guest cells is known as emperipolesis[15]. Emperipolesis is different from phagocytosis, during which morphologically altered cells or cell debris are internalized and disposed of[16]. The interaction between lymphocytes and motoneurons in the course of acute EAE shares features with both emperipolesis and phagocytosis.

Treatment with AMPA antagonists NBQX, MPQX, GYKI52466 and GYKI53773, begun at the onset of neurological decline, caused a profound reduction in the neurological deficits of acute EAE. The beneficial clinical effects of AMPA antagonists cannot be attributed to anti-inflammatory or immunomodulatory actions, as NBQX had no effect on neuroinflammation (perivascular cuffs), was effective in adoptive transfer EAE, and NBQX and MPQX did not inhibit the concanavalin A-induced proliferation of T lymphocytes. The reduction of neurological sequelae of acute EAE by AMPA antagonists and the beneficial effects of NBQX in chronic relapsing EAE substantiate the possibility of the involvement of glutamate in the disease pathogenesis.

Increased concentrations of glutamate in the cerebrospinal fluid of patients suffering from multiple sclerosis have been re-

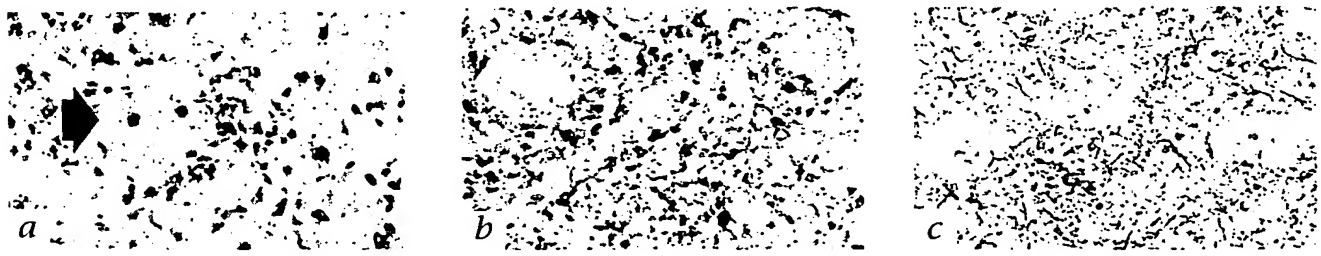

Fig. 3 Light immunomicrographs showing that T lymphocytes but not macrophages/microglia or astrocytes invade motoneurons. *a*, A cell staining positive with OX34 specific for CD2 on T lymphocytes is present inside a motoneuron (arrow). *b*, Cells staining positive with ED1, which recognizes a lysosomal membrane-related antigen on macrophages/microglia, are present outside motoneurons and do not show propensity to invade them. *c*, Cells positive for glial fibrillary acidic protein (astrocytes) are present outside motoneurons and do not invade them. Similarly, cells positive for OX33 (specific for CD45RA on B lymphocytes) or clone 10/78 (specific for CD161 on natural killer lymphocytes) do not invade motoneurons. Original magnification, × 360; counterstained with hematoxylin.

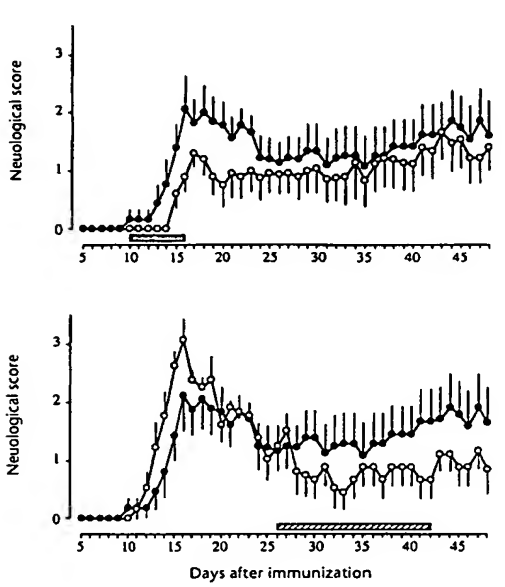

Fig. 4 Effect of AMPA/kainate antagonist NBQX on chronic relapsing EAE in mice. *a*, Mice were given 30 mg/kg NBQX (n = 10; open circles) or vehicle (n = 9; ●) intraperitoneally twice daily on days 10–16 after immunization (stippled bar). *b*, Mice were given 30 mg/kg NBQX (n = 7; ○) and vehicle (n = 10; ●) intraperitoneally once daily on days 26–42 after immunization (hatched bar). Treatment with NBQX on days 10–16 after immunization significantly improved neurological outcome ($F_{10-48}(1,38)=9.21$, $P < 0.001$, repeated measures analysis of variance); delayed treatment with NBQX on days 26–42 after immunization also significantly improved neurological status in mice ($F_{28-48}(1,20)=2.76$, $P < 0.05$). Data represent means ± s.e.m.

ported[17]. The activity of glutamine synthetase and glutamate dehydrogenase is compromised in astrocytes during EAE, affecting the reuptake of glutamate[18]. AMPA receptors mediate the death of oligodendrocytes *in vitro*[19], whereas microinfusions of kainate into the optic tract in rodents produce demyelination[20]. These observations indicate that glutamate may target both non-neuronal and neuronal elements in the spinal cord to induce neurological decline during EAE. Thus, the clinical and morphological changes in EAE may be attributed to the 'unleashed' toxic action of glutamate or related agents on various cellular populations in the spinal cord.

These conclusions may be relevant for the therapy of multiple sclerosis and perhaps other demyelinating disorders. The treatment of multiple sclerosis relies exclusively on immunosuppressive agents[3]. Interferon-β has proven efficacious in the relapsing/remitting form of multiple sclerosis[3]. Our results indicate that therapeutic strategies for multiple sclerosis should be complemented by neuroprotective measures to reduce neurological disability and neuronal injury.

Methods

Induction of EAE. Lewis rats (200–220 g in body weight; Charles River, Manson, UK) were immunized subcutaneously in the dorsal surface of each hind foot with 50 µl inoculum containing 50 µg guinea pig myelin basic protein, prepared according to a published method[21,22] and emulsified in Freund's complete adjuvant containing 5.5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, Michigan). 'Sham-immunized' rats received Freund's complete adjuvant containing *M. tuberculosis* alone "subcutaneously".

Neurological assessment. Neurological deficits were monitored daily during the course of EAE by a single observer 'blinded' to treatment status, before administration of vehicle or drugs, to ensure that the motor side effects of therapy with AMPA antagonists such as sedation or reduction of muscle tone did not interfere with disability scores. The following scoring system was used to grade neurologic impairment: 0, no detectable changes in muscle tone and motor behavior; 1, flaccid tail; 2, impairment of righting reflex and/or loss of muscle tone in hindlimbs; 3, complete hindlimb paralysis; 4, paraplegia; and 5, death. During the observation period, rats were housed in pairs in environmentally controlled conditions (6:00–18:00, 12-hour light/dark cycle; 24–25 °C) and were permitted free access to food and water. Body weight was monitored daily with a Sartorius model U 6100 balance.

Morphology. Rats were anesthetized with an overdose of pentobarbital and perfused with a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (for combined light and electron microscopy). Coronal sections of the lumbar spinal cord were cut 10–15 µm thick, mounted on a glass slide and stained with hematoxylin and eosin or cresyl violet. For electron microscopy, the tissue was processed in osmium tetroxide and uranyl acetate, dehydrated in a series of increasing ethanol concentrations, 'cleared' in propylene oxide, embedded in araldite and viewed by transmission electron microscope. For light microscopy, 'semithin' coronal sections of the lumbar spinal cord 1 µm in thickness were cut and stained with toluidine blue.

Quantification of neuronal density in the spinal cord. To provide an estimate for possible neuronal loss in the spinal cord over the period of up to 16 d after immunization, numerical densities of neurons in the ventral and dorsal horns and in the intermediate zone of the lumbar cord were determined using a stereologic dissector[23,24]. An unbiased counting frame (0.1 mm x 0.1 mm; dissector height, 0.015 mm) and a high-aperture objective (x100) were used for the sampling[24]. Normal neurons were identified by the presence of the typical nuclei with clear nucleoplasm and distinct nucleolus surrounded by cytoplasm. An arbitrary horizontal line connecting the lateral ends of the spinal cord and crossing the ventral edge of the central commissure was considered the junction between the intermediate zone and the ventral horns. An arbitrary parallel horizontal line crossing the dorsal edge of the central commissure was considered the junction between the intermediate zone and the dorsal horns. The gray matter of the ventral horns contains motoneurons controlling function of the limbs and trunk, and interneurons. The intermediate zone contains sensory neurons projecting towards the cerebellum, preganglionic autonomic neurons and interneurons. The dorsal horns contain sensory projection neurons connecting supraspinal centers.

Immunohistochemistry. Immunohistochemical analysis of the lumbar spinal cord was done in rats with scores 3–4 at the peak of the disease course. The following murine monoclonal antibodies (all from Serotec, Raleigh, North Carolina, except 5.2E4: Institute of Neurology, London, UK) were used at the following dilutions to examine resident and infiltrating cells in the spinal cord: OX34 (1:500), specific for CD2 on T lymphocytes; OX33 (1:100), specific for CD45RA on B lymphocytes; clone 10/78 (1:100), specific for CD161 on natural killer lymphocytes (1:100); ED1 (1:500), which recognizes a lysosomal membrane-related antigen on rat macrophages/microglia; OX-42 (1:3,000), which binds the complement receptor 3 on microglia/macrophages; and 5.2E4 (1:1,000), for glial fibrillary acidic protein staining of astrocytes. Freshly frozen spinal tissue, cut into sections 10 µm in thickness, was fixed in ethanol, incubated with the primary antibody, and washed before a biotinylated rat-absorbed antibody against mouse IgG (1:200 dilution; Vector Laboratories, Burlingame, California) was added. Peroxidase-labeled avidin–biotin complex (ABC; Vector Laboratories, Burlingame, California) was added to the sections, and peroxidase activity was detected in a 3,3'-diaminobenzidine (Sigma) solution in phosphate-buffered saline containing 0.01% hydrogen peroxide. Rinsed sections were counterstained in hematoxylin, dehydrated in a series of increasing alcohol concentrations, 'cleared' in xylene and mounted on glass slides. Sections with no primary antibody were included with each staining, as controls.

Treatment regimen. Rats were treated with the following competitive AMPA/kainate antagonists intraperitoneally twice daily for 7 days starting on day 10 after immunization: 3–30 mg/kg NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline) or 2.5–10 mg/kg MPQX ([1,2,3,4-tetrahydro-7-morpholin-yl-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methylphosphonate). This treatment regimen was chosen to ensure that sufficient concentrations were achieved in the brain to interact with AMPA/kainate receptors[12,13]. Rats received 40 mg/kg GYKI52466 (non-competitive AMPA antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5H-2,3-benzodiazepine) intraperitoneally at 0, 6, 12, 18 and 24 h on day 10 after immunization. This treatment regimen allows for maintenance of relevant concentrations of GYKI52466 in the brain for several days[14]. Rats were treated with 30 mg/kg of the non-competitive AMPA antagonists GYKI52466 and GYKI53773 ((-)1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-4,5-dihydro-3-methylcarbamoyl-2,3-benzodiazepine) intraperitoneally twice daily for 7 days starting on day 10 after immunization. This treatment regimen was used to ensure that relevant concentrations in the brain to interact with AMPA dependent ion channels were reached[14]. NBQX, MPQX, GYKI52466, GYKI53773 and vehicle were administered in a volume of 0.5 ml/100 g body weight.

Adoptive transfer of EAE. Ten days after being immunized with guinea pig myelin basic protein, Lewis rats were killed and their spleens were removed. Splenic lymphocytes were cultured for 72 h at 37 °C in an atmosphere of 5% CO_2 and 95% O_2 at a concentration of 2×10^6/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 µM 2-mercaptoethanol and 1 mg/ml indomethacin with 10 µg/ml myelin basic protein. Collected lymphocytes were washed in unsupplemented RPMI 1640, and 4×10^7 cells were transferred intraperitoneally to non-immunized recipients.

Quantification of neuroinflammation. Sections of rat brainstem (anterior–posterior, –7.0 to –15.0; ventral, –8.60) (ref. 25) 10 µm in thickness were stained with hematoxylin and assessed for the presence of perivascular cuffs by an observer 'blinded' to sample identity. The following scoring system was used to grade histopathological changes induced by inflammation: 0, no detectable changes; 1, perivascular inflammation of up to three cell layers; 2, perivascular inflammation of more than three cell layers; and 3, parenchymal cell infiltrates. The histopathological score was calculated by adding all scores for lesions detected in a given section, and the mean of two sections was used for statistical analysis.

Quantification of immunosuppresion in an *in vitro* stimulation assay. Splenic lymphocytes from Lewis rats were incubated at a concentration of 1×10^6 cells/ml in RPMI 1640 modified medium with 1 µg/ml concanavalin A and increasing concentrations of AMPA antagonists (0.01, 1, 10 and 100 µM) or dexamethasone (0.001, 0.01, 0.1 and 1 µM). Then, 96 h after the addition of the stimulating mitogen, 1 µCi ^{3}H-thymidine was added for an additional 6 h, then the cells were collected and incorporated radioactivity was measured. Lymphoproliferation in response to the lectins phytohemagglutinin or concanavalin A requires engagement of the T-lymphocyte receptor and synthesis of the T-lymphocyte growth factor interleukin-2. Agents that inhibit lymphoproliferation, such as dexamethasone, show immunosuppresive action.

Induction of chronic relapsing EAE. Spinal cords from Biozzi mice (Ab/H, H-2^{ag}, body weight 20–22 g; Harlan UK, Bicester, UK) were homogenized and freeze-dried. Lyophilized spinal cord homogenate was reconstituted in phosphate-buffered saline to a final concentration of 6.6 mg/ml. Incomplete Freund's adjuvant was supplemented with *M. tuberculosis* (H37Ra; and *M. butyricum* (both from Difco, Detroit, Michigan) in a ratio of 8:1. Biozzi mice were immunized subcutaneously in the flank at three sites on day 0 and day 7 with 0.3 ml of the emulsion (1 mg spinal cord homogenate and 60 µg of combined *M. tuberculosis* and *M. butyricum*). In addition, mice were injected intraperitoneally with 200 ng *Bordetella pertussis* toxin (2 µg/ml in phosphate-buffered saline; Calbiochem, La Jolla, California) immediately and 24 h after immunization with neuroantigens. Mice were treated intraperitoneally with 30 mn/kg NBQX twice daily on days 10–16 after immunization or once daily on days 26–42 after immunization. Neurological deficits were monitored daily by an observer 'blinded' to treatment status, before administration of vehicle or drugs. The following scoring system was used to grade neurological impairment: 0, no detectable changes; 1, flaccid tail; 2, impairment of righting reflex and/or loss of muscle tone; 3, complete hindlimb paralysis; 4, paraplegia; and 5, death. During the observation period, mice were housed in pairs in environmentally controlled conditions (6:00–18:00, 12-hour light/dark cycle; 24–25 °C) and were permitted free access to food and water.

Statistics. Data were analyzed statistically by means of analysis of variance, Student's *t*-test, Mann-Whitney U test, χ^2 test, and Fisher's protected least-significant-difference test.

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Glutamate excitotoxicity in a model of multiple sclerosis

DAVID PITT[2], PETER WERNER[1,3] & CEDRIC S. RAINE[1,2,4]

Departments of [1]Neurology, [2]Pathology and [4]Neuroscience, F-121N, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York, 10461, USA
[3]Dept. of Neurology, Beth Israel Medical Center, 10 Union Square East, New York, New York 10003, USA
D.P and P.W. contributed equally to this study.
Correspondence should be addressed to P.W.; email: pwerner@aecom.yu.edu.

Glutamate excitotoxicity mediated by the AMPA/kainate type of glutamate receptors damages not only neurons but also the myelin-producing cell of the central nervous system, the oligodendrocyte[1]. In multiple sclerosis, myelin, oligodendrocytes and some axons are lost as a result of an inflammatory attack on the central nervous system[2]. Because glutamate is released in large quantities by activated immune cells[3], we expected that during inflammation in MS, glutamate excitotoxicity might contribute to the lesion. We addressed this by using the AMPA/kainate antagonist NBQX to treat mice sensitized for experimental autoimmune encephalomyelitis, a demyelinating model that mimics many of the clinical and pathologic features of multiple sclerosis. Treatment resulted in substantial amelioration of disease, increased oligodendrocyte survival and reduced dephosphorylation of neurofilament H, an indicator of axonal damage[4]. Despite the clinical differences, treatment with NBQX had no effect on lesion size and did not reduce the degree of central nervous system inflammation. In addition, NBQX did not alter the proliferative activity of antigen-primed T cells *in vitro*, further indicating a lack of effect on the immune system. Thus, glutamate excitotoxicity seems to be an important mechanism in autoimmune demyelination, and its prevention with AMPA/kainate antagonists may prove to be an effective therapy for multiple sclerosis.

For this study, we determined whether glutamate excitotoxicity due to increased extracellular glutamate is important in damage to oligodendrocytes and axons in experimental autoimmune encephalomyelitis (EAE). As oligodendrocytes have only the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)/kainate type of excitatory glutamate receptor and are exquisitely vulnerable to glutamate excitotoxicity[1,5], we treated mice subjected to EAE with NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline-2,3-dione), an AMPA/kainate antagonist. We induced EAE by adoptive transfer of myelin basic protein (MBP)-specific lymph node cells from previously immunized donor SJL/J mice into naive recipients. In two independent experiments, adoptively sensitized mice ($n = 19$) received three subcutaneous injections per day of 300 μg NBQX in 200 μl phosphate-buffered saline (PBS) beginning day 5 after transfer and continuing until the end of the experiment (day 15). Control mice with EAE ($n = 18$) received vehicle (PBS) only. A clinical score from 0 (healthy) to 5 (moribund or dead) was assigned to assess neurologic impairment[6]. Treatment with NBQX led to a highly significant reduction ($P < 0.01$) in clinical impairment compared with treatment with vehicle alone (Fig. 1). The difference in clinical score was significant ($P < 0.05$) by day 3 after disease onset (day 7 of treatment), and continued to increase until the time of sampling ($P < 0.01$), attaining a final mean difference between experimental and vehicle-treated groups of 1.8 clinical score points. Histologic examination of the neuraxes of two representative mice in each group showed they had similar degrees of inflammation, indicating that NBQX did not function by modulating immune responsiveness. Thus, we assessed the effect of NBQX on the immune response by testing the proliferation of lymph node cells *in vitro* in the presence of NBQX. We stimulated cultured lymph node cells from MBP-sensitized mice with 50 μg/ml MBP and determined their proliferation rates in the presence or absence of NBQX. In three independent experiments (six samples each), there was no significant difference in ^{3}H-thymidine incorporation by NBQX-treated cells (counts per minute: unstimulated, 352 ± 63; MBP, 2,200 ± 441) and control cells (counts per minute: unstimulated, 261 ± 19; MBP, 2,610 ± 567). In addition, pretreatment of MBP-activated lymph node cells with NBQX for 72 hours did not reduce their efficacy in inducing EAE when injected into naive mice but resulted in the usual form of EAE (ref. 6). Thus, NBQX substantially ameliorated the clinical course of EAE, indicating that glutamate excitotoxicity was responsible for a substantial portion of the clinical impairment in vehicle-treated mice with EAE. This effect was not due to a change in T-cell function or central nervous system (CNS) inflammation.

The principal cellular target in multiple sclerosis (MS) is the oligodendrocyte. To evaluate the role of glutamate excitotoxicity in the loss of oligodendrocytes during EAE, we examined oligodendrocytes in detail by immunohistochemistry of lumbar spinal cord tissue from representative mice from the NBQX- and vehicle-treated groups. We immunolabeled frozen sections with antibody against CNPase and counted the total number of oligodendrocytes within the dorsal columns in 20–30 transverse sections (Fig. 2*a*). In mice with EAE, dorsal columns commonly have inflammatory lesions centered on midline vessels, and this feature allowed us to reproducibly evaluate the effect of NBQX on the lesion area. Within this well-defined region, the number of oligodendrocytes was significantly less ($P < 0.001$) in vehicle-treated EAE than in normal, age-matched mice. However, the number of oligodendrocytes in mice with EAE treated with NBQX was only slightly less than that in normal mice (Fig. 2*b*). Expressed as percentage of cell loss in comparison to normal mice, 23% of oligodendrocytes ($P < 0.001$) were lost in mice with vehicle-treated EAE, compared with only 9% in NBQX-treated mice ($P > 0.05$) (Fig. 2*c*). Overall, more than 60% of the total oligodendrocyte loss in vehicle-treated mice was prevented with treatment with NBQX. Thus, our results indicate that AMPA/kainate receptor-mediated glutamate excitotoxicity is a chief factor in oligodendrocyte death in EAE.

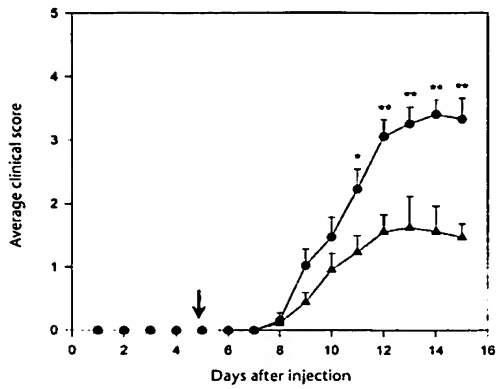

Fig. 1 Clinical course of adoptive transfer EAE: Effect of treatment with NBQX. SJL mice were injected with MBP-activated cells. Starting on day 5 after immunization (downward arrow), mice were treated with NBQX or vehicle (PBS) until day 15 (day 10 of treatment). Data represent means ± s.e.m. from two independent experiments, with a total of 18 mice until day 12, and 9 mice for days 13–15. *, $P < 0.05$ and **, $P < 0.01$, vehicle-treated compared with NBQX-treated, Student's unpaired, two-tailed *t*-test.

Axonal damage is another important feature of MS lesions and has been shown to increase with lesion activity[4,7]. A recently exploited immunohistochemical marker of demyelinated and dystrophic axons in MS, the presence of abnormal dephosphorylation of heavy chain neurofilament H (NF-H), has been used to assess axonal damage[4]. Using the same technology, with western blot analysis, we showed that as in MS, the central nervous systems of mice with EAE had a large increase of abnormally dephosphorylated NF-H, demonstrable both structurally (Fig. 3*a*) and semi-quantitatively (Fig. 3*b* and *c*). 'Terminal' EAE mice (that is, grade 5) had large amounts of abnormally dephosphorylated NF-H, whereas normal mice had much smaller amounts (Fig. 3*b* and *c*). A background level of reactivity in the spinal cords of normal mice was consistent with the presence of dephosphorylated NF-H in neuronal cell bodies and some large-diameter axons[8]. Treatment of EAE with NBQX resulted in a substantial reduction of abnormally dephosphorylated NF-H compared with that in vehicle-treated mice (Fig. 3*b* and *c*), indicating involvement of glutamate excitotoxicity in the axonal changes. This is in agreement with the well-known vulnerability of neurons to glutamate excitotoxicity and the reported presence of ionotropic glutamate receptors on myelinated axons[9]. Thus, treatment of mice with EAE with the AMPA/kainate antagonist NBQX led to a considerable amelioration of clinical outcome, which corresponded pathologically to a reduced loss of oligodendrocytes as well as diminished axonal damage.

These observations relate directly to oligodendrocyte death and axonal damage in MS, the underlying mechanisms of which are believed to be immune-associated. Activated immune cells present in the inflammatory infiltrates in CNS lesions produce cytotoxic factors such as tumor necrosis factor α (ref. 10), matrix metalloproteinases[11], active oxygen species[12] and autoantibodies[13], and may also kill by direct cell-to-cell contact. During inflammation, glutamate is produced and released into the extracellular space[3] by activated leukocytes and microglia. However, this topic has received little attention. Within the CNS, increase in extracellular glutamate during inflammation may be further enhanced by reduction of the glial glutamate-metabolizing enzymes, glutamate dehydrogenase and glutamine synthetase, as shown in EAE (ref. 14). Accordingly, increased glutamate levels have been found in the cerebrospinal fluid of patients with CNS inflammatory conditions, such as acute encephalitis, meningitis and MS (refs. 15,16). An increase in extracellular glutamate, the main excitatory neurotransmitter in the CNS, can have potentially serious consequences, as it is capable of precipitating excitotoxic cell death by overstimulation of ionotropic glutamate receptors[1,17], two types of which are recognized: NMDA (N-methyl D-aspartate) and AMPA/kainate. Both types are found on neurons, whereas glial cells have only AMPA/kainate receptors[1,5].

In the context of EAE, given the plethora of other cytotoxic factors known to be present in EAE lesions, it was unexpected that more than 60% of the oligodendrocyte loss in our experiments could be attributed to glutamate excitotoxicity. It is unlikely that

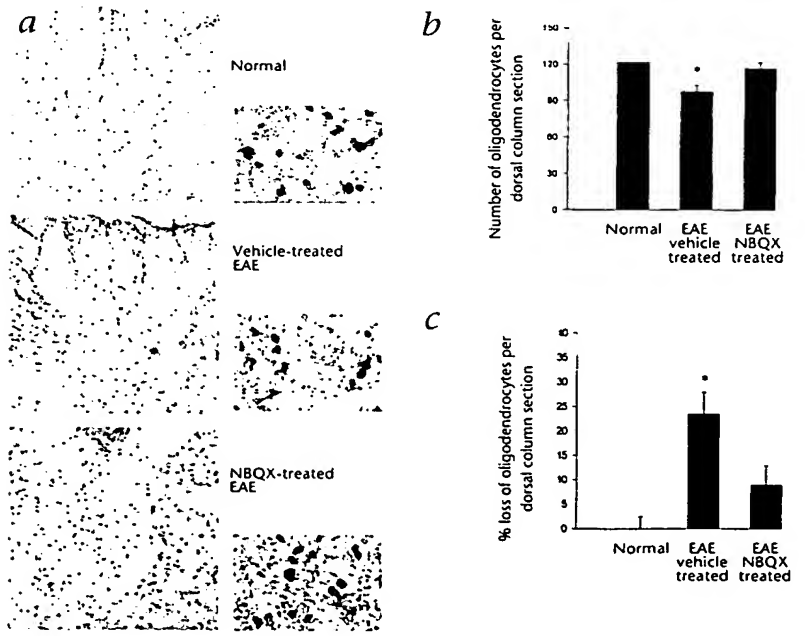

Fig. 2 CNPase-immunoreactive oligodendrocytes in lumbar spinal cord sections. ***a***, Transverse sections of dorsal columns immunolabeled with antibody against CNPase (amplified with ABC and visualized with HRP/DAB; hematoxylin counterstaining). Original magnifications, × 30 (left) and × 100 (right). ***b***, Average number of oligodendrocytes per transverse section of dorsal columns. Each bar represents two representative mice from the normal, vehicle-treated and NBQX-treated groups, with 20–30 sections analyzed per mouse. ***c***, Oligodendrocyte loss in treated and untreated EAE mice and normal mice. Data are expressed as percentage of cells lost per dorsal column and represent means ± s.e.m. *, $P < 0.001$, vehicle-treated compared with normal, and *P*, not significant, vehicle-treated compared with NBQX-treated, ANOVA followed by post-hoc Tukey.

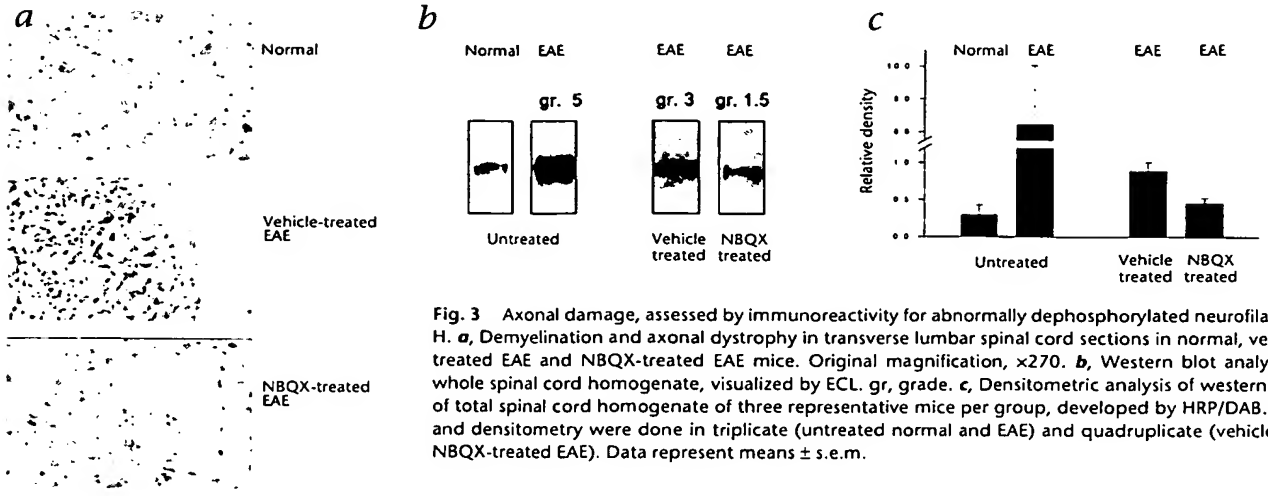

Fig. 3 Axonal damage, assessed by immunoreactivity for abnormally dephosphorylated neurofilament H. ***a***, Demyelination and axonal dystrophy in transverse lumbar spinal cord sections in normal, vehicle-treated EAE and NBQX-treated EAE mice. Original magnification, ×270. ***b***, Western blot analysis of whole spinal cord homogenate, visualized by ECL. gr, grade. ***c***, Densitometric analysis of western blots of total spinal cord homogenate of three representative mice per group, developed by HRP/DAB. Blots and densitometry were done in triplicate (untreated normal and EAE) and quadruplicate (vehicle and NBQX-treated EAE). Data represent means ± s.e.m.

blockade of glutamate receptors in the lesion center would prevent cell death; however, the degree of protection in the surrounding parenchyma indicated a substantial bystander effect, akin to the spreading excitotoxic damage described in stroke[18], in which AMPA/kainate receptor antagonists have received considerable attention[19]. Although the loss of oligodendrocytes in chronic EAE parallels that in MS (ref. 7), we have now quantified the phenomenon in evolving lesions and shown that depletion of oligodendrocytes can be reduced, even during the acute phase. The extent of protection against oligodendrocyte loss demonstrated here indicates glutamate excitotoxicity may be one of the main mechanisms of oligodendrocyte demise in EAE, and perhaps in MS. Our results are supported by a recent report demonstrating that NBQX protects against white matter damage in spinal cord crush injury, a situation in which large amounts of glutamate are released into the extracellular space[20]. Whether changes in glutamate production can be detected in the cerebrospinal fluid of EAE-affected mice is now being investigated using a published method[21], and this forms part of an ongoing study on glutamate metabolism in these conditions (P.W. *et al.*, manuscript in preparation).

Dephosphorylation of NF-H has provided a useful quantitative molecular marker for the severity of EAE, emphasizing the association between axonal involvement and neurologic dysfunction and confirming recent immunohistochemical results with MS lesions[4]. This raises the possibility that retrograde degeneration of neurons in demyelinating diseases[22] may be related to excitotoxic events. The beneficial effects of NBQX on the course of EAE seemed to be due to axonal sparing and oligodendrocyte protection in the absence of a detectable effect upon inflammation, a new concept in this MS paradigm.

In conclusion, we have shown glutamate excitotoxicity mediated by AMPA/kainate receptors to be important in CNS damage in EAE and by extrapolation, possibly in MS. Thus, AMPA/kainate antagonists, now being tested in stroke patients, may afford promising avenues in the treatment of this devastating human disease.

Methods

Induction of EAE. Female adult SJL/J mice 4–6 weeks old (Jackson Laboratories, Bar Harbor, Maine), were housed in a light- and temperature-controlled environment in accordance with National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Myelin basic protein (MBP; Sigma) was dissolved in sterile PBS at a concentration of 8 mg/ml and emulsified with an equal volume of incomplete Freund's adjuvant supplemented with 6 mg/ml *Mycobacterium tuberculosis* (Difco, Detroit, Michigan). Ten days after antigen was injected into the flanks of SJL/J mice, lymph node cells were obtained from draining lymph nodes, cultured and grown for 4 d in the presence of 50 μg/ml MBP, and were subsequently injected intravenously into syngeneic mice at a dose of 3×10^7 cells/mouse. Onset of disease occurred usually after 7–9 d, and mice were graded daily according to a standard clinical index[15] of 0–5 by an individual 'blinded' to mouse identity.

Treatment of EAE. NBQX (Tocris, Ballwin, Missouri) was administered in three daily subcutaneous injections of 300 μg in 200 μl PBS for the duration of the experiment (7 and 10 d). Control mice were treated with PBS alone.

Neuropathology. After 7 and 10 d of treatment, respectively, mice from the vehicle- and NBQX-treated groups were perfused with ice-cold PBS or glutaraldehyde and the CNS was prepared for frozen or epoxy sections, respectively. Epoxy sections 1 μm in thickness from nine levels of the neuraxis (optic nerve, cerebrum, brainstem and spinal cord at C7, Th2, L2, L5, L6 and S1) were stained with toluidine blue and examined by light microscopy, by an individual 'blinded' to the coded sample identity. Frozen sections from lumbar spinal cord (10μm) were acetone-fixed and immunolabeled using the avidin:biotinylated enzyme complex technique (ABC; Vector Laboratories, Burlingame, California). The following antibodies were used: antibody against CNPase (Sigma) at a dilution of 1:80; and antibody against non-phosphorylated neurofilament-H (SMI 32; Sternberger Monoclonal, Lutherville, Maryland) at a dilution of 1:10,000. Sections were viewed with a Zeiss Photomicroscope III, and cells positive for antibody against CNPase were counted using an ocular graticule.

T-cell proliferation assay. Lymph node cells from MBP-immunized mice (described above), were grown in 96-well plates at a concentration of 0.5×10^6 cells per well in 200 μl medium containing 50 μg/ml MBP in the presence or absence of 5 μM NBQX (Tocris, Ballwin, Missouri). After 3 d, cells were 'pulsed' with 1 μCi/ml ^{3}H-thymidine for 12 h before being collected onto glass fiber mats. The incorporated radioactivity was measured in a scintillation counter.

Western blot analysis. PBS-perfused spinal cords were homogenized in 8.5 M urea containing 2 mM PMSF. Samples were denatured in sampling buffer for 2 min at 100 °C, then separated by 10% SDS–PAGE and blotted onto a PVDF membrane. After this transfer, the membrane was blocked for 10 min in 5% fat-free instant milk. Immunodetection was accomplished by incuba-

tion overnight at 4 °C with primary monoclonal antibody SMI 32 (1:10,000 dilution) and, as a standard, antibody against tubulin (1:1,000 dilution; Sigma). After being washed, the blots were incubated with horseradish peroxidase (HRP)-labeled goat antibody against mouse IgG1 (1:500 dilution; Southern Biotechnology, Birmingham, Alabama), for 1 h and then washed. Blots were developed with an ECL kit (Amersham, Piscataway, New Jersey). Alternatively, biotinylated secondary antibody was used and the avidin:biotinylated enzyme complex (ABC) technique was used, with diaminobenzidine (DAB) as substrate.

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